UNIVERSIDAD AUTÓNOMA DE MADRID

DEPARTMENT OF MOLECULAR BIOLOGY

Maintenance of epidermal architecture by CLASP2 and p120-catenin

Marta Násila Shahbazi Alonso

Madrid, July 2013

UNIVERSIDAD AUTÓNOMA DE MADRID

DEPARTMENT OF MOLECULAR BIOLOGY

Maintenance of epidermal architecture by CLASP2 and p120-catenin

Doctoral thesis submitted to the Universidad Autónoma de Madrid for the degree of Doctor of Philosophy by M. Sci. in Molecular Biomedicine,

Marta Násila Shahbazi Alonso

Thesis Director

Dr. Mirna Pérez-Moreno

Epithelial Cell Biology Group

Cancer Cell Biology Programme

Spanish National Cancer Research Centre

Dr. Mirna Pérez-Moreno, head of the Epithelial Cell Biology Group in the Spanish National Cancer Research Centre (CNIO),

CERTIFIES

That Ms Marta Násila Shahbazi Alonso, Master in Molecular Biomedicine by the Universidad Autónoma de Madrid, has completed her Doctoral Thesis **"Maintenance of epidermal architecture by CLASP2 and p120-catenin"** and meets the necessary requirements to obtain the PhD Degree in Molecular Biosciences. To this purpose, she will defend her Doctoral Thesis at the Universidad Autónoma de Madrid. The Thesis has been carried out under my direction and hereby I authorize it to be defended to the appropriate Thesis Tribunal.

I hereby issue this certificate in Madrid on April 29th 2013.

Mirna Pérez-Moreno

PhD Thesis Director

Isabel Correas Hornero

PhD Thesis Tutor

Acknoledgments

He escrito estos agradecimientos en múltiples ocasiones en mi cabeza a lo largo de mis cinco años de experiencias en el CNIO. Y de repente, el fin de una etapa llega, la tesis es ya una realidad y he de plasmar en unas líneas mi gratitud hacia todas las personas que han sido parte de mi vida durante estos años y han contribuido a que yo hoy sea una candidata a doctora.

Mirna, comenzamos esta aventura juntas. Montar el laboratorio de cero, las células diferenciadas o los ratones sin fenotipo son cosas que difícilmente olvidaremos. Y a pesar de los duros comienzos, la experiencia ha sido verdaderamente positiva. La madurez, la independencia y el aprendizaje que he obtenido durante todo este tiempo son irremplazables. Gracias por tu preocupación, por tu dedicación y por tus enseñanzas. Gracias por no ser sólo una jefa sino una mentora.

Carolina, la mejor maestra de laboratorio que se pueda encontrar. He aprendido a hacer experimentos a tu lado. Gracias por los clonajes, las purificaciones o las mil y una dudas de todos estos años. Pero sobre todo, gracias por ser tan buena compañera. ¡Será duro volver al "bench" si tenerte al lado! Ljiljana, "my secretary": you are much more than a lab mate! Thanks for the laughs, the trips, CNIOSA and all these years together. Sharing with you so many great things has created a bond that for sure will survive. Donatello, el cromosoma Y que todo laboratorio necesita. Gracias por traer diversión a nuestra rutina diaria. "I like!". Francy, ha sido un placer hacer experimentos a tu lado estos últimos años. Gracias por tu ayuda y por estar siempre disponible. Sin lugar a dudas, ¡somos un team de "pink catwomen"! Lucho, tú fuiste mi hermano mayor de aquellos primeros años en el laboratorio. Gracias por preocuparte siempre de "la niña".

From outside the ECB lab, I am thankful to other people from CNIO, especially to the members of the Cancer Cell Biology Programm because we have shared more than just space.

Erwin, thank you for teaching us that one must speak up for the things that are right. Thank you for caring about all the members of the program, for your support and for always having the door open. And the take home message is clear: science is fun!

Juan, mi modelo a seguir del programa. Te admiro como científico y como amigo. Helia, thank you for been always willing to help. From printing the posters, to designing an experiment or making a figure, you have always been there. Latifa, thank you for sharing with me your "rituals". María, gracias por hacer que el programa funcione. Özge, thank you for your smile, your positive attitude towards life and your care. You are a sweetheart. Thanks to Stefanie, the top model of the program! Eva, empezamos juntas y terminamos juntas. ¡Me ha encantado compartir estos años contigo! Mohamad, you are the sunshine of my life. Krishna, the little kid of the family: you are pure kindness! Many thanks to all the good friends that I have made during these years at CNIO: Enrico,

Sabela, Karsten, Han, Paula and Yev. Iva, since that day we sat together in the bus our friendship has become stronger. I am so glad to have you as a friend!

Muchas gracias a la chicos de mi unidad preferida: Ximo, Manu, y especialmente Diego por toda su paciencia y ayuda cuantificando lo incuantificable. Gracias a Flor, por cuidar tan bien de nuestros ratones.

A very special thanks goes to the members of my “second lab” in New York. Gregg, you are my role model as a scientist. Thank you for your continuous support, for teaching me so much and sharing with me your enthusiasm about science. Guilherme, no matter how many oceans are between us, or how many months pass by, an invisible bond will keep us together. I maybe just need another aliquot of secondary antibody…! Nagendran, my dear, thank you for been the best friend one could imagine. Let’s live closer in the future!! Susumu, you know I will always miss our conversations in the microscope room. Wakam and Sara, many thanks for all your help during my stay. Francesca, thank you for all the scientific discussions and showing me some of the NY city fun!

Muchas gracias a los miembros de mi comité de tesis: Marcos Malumbres, Amparo Cano y Marisol Soengas. Vuestros comentarios, sugerencias y apoyo durante estos años han sido fundamentales para que mi proyecto llegara a buen puerto. Special thanks to Terry Lechler for his continuous input, which has been fundamental to improve both my thesis and my manuscript.

En el mundo no científico, muchas personas han estado a mi lado durante estos 5 años, apoyándome en todo momento, y preguntándome: Marta, ¿qué tal tus células? o ¿cómo van los ratones?

Gracias a mis amigas de toda la vida, porque nos hemos mantenido unidas en todo momento, y eso es algo que nunca perderemos. Alicia (aunque deberías ir en el apartado de hermana), gracias por escucharme siempre y tener además un buen consejo que dar. Gracias por los inolvidables años en los que hemos vivido juntas. Laura, la mejor compañera de mesa durante tantos años de instituto, verdad extrema, ¡siempre serás la primera que me invitó a su casa a ver Scream! Ana José, hermanas de hermanos, gracias por todo tu cariño y amor. Scully, aunque ahora estés lejos, te siento cerca. Gracias a Edu por cuidar tan bien de Ana José y soportar todas nuestras veladas de mujeres al borde de un ataque de nervios.

Mil gracias a la mejor amiga que uno pueda imaginarse: Cris. ¡Gracias a Dios que me acerqué a ti aquel día en la universidad y os acompañé a la biblioteca! Hemos vivido juntas todos los acontecimientos más importantes de nuestras vidas en los últimos 10 años y seguiremos haciéndolo en el futuro. Gracias a José Víctor por todo su cariño y por hacerme reir tantísimo. Los 20 días que

pasamos juntos en NY fueron realmente inolvidables. Gracias a David Ruano, siempre dispuesto a escuchar mis frustraciones y avances científicos, y a José Zapatero, el más interesado en mis descubrimientos. María Alonso, gracias por acogerme en Ámsterdam en una época crítica y por haberte convertido en una buena amiga. Amparo, gracias por acordarte siempre de mí, sobre todo cuando las cosas no van tan bien. Gracias a Luis Toledo, por transformar mi vida de la noche a la mañana.

Gracias a mis profesores de Biología del instituto, Santiago Barahona y Nacho Saz-Orozco, porque me abrieron las puertas de mi verdadera vocación: la biólogía.

Mi familia siempre ocupará el lugar más especial de mi corazón. Mamá y papá, desde pequeños nos inculcásteis el espíritu del esfuerzo y del trabajo: lo que importaba no era la nota, sino el tesón personal y la dedicación, cualidades que han sido fundamentales para desarrollar un trabajo científico. A vosotros os debo lo que soy y lo que he conseguido. Gracias por estar siempre a mi lado y por enseñarme ante todo a ser buena persona. Carlos, me hace feliz que sigamos caminos paralelos en la vida. Los dos científicos… ¡Podrás seguir durante muchos años preguntándome cuándo voy a publicar el artículo!

Gracias a abuelito por inculcarme la importancia del saber y de la educación: “primero la carrera, después los chicos”. Gracias a abuelita, la abuela más orgullosa de su rosina. Tía Tere, mi segunda mamá, te quiero muchísimo. Tía Pepita, tanto Carlos como yo seguimos tus pasos. Gracias por tus consejos, por tu apoyo y por ayudarme a ver el camino. Gracias por ser un modelo a seguir. Gracias a Ramón y a mi primo favorito Javi Gang.

تشکر به خانواده ایرانی من. دلم برای شما تنگ شده.

Nella vita, come nello scacchi, la cosa più importante è chiedersi perché. Mirco, tu sei la risposta al perché della mia vita.

Gracias a todos.

Abstract

Adherens Junctions are cadherin-based structures fundamental for the maintenance of cell-cell adhesion, and thus preservation of proper tissue architecture and function. To do so, they establish connections with the cytoskeleton, both with actin filaments and microtubules, enabling cells to respond to injury and external stimuli as a coordinated tissue.

Previous findings have illustrated connections between cadherins and microtubule minus and plus-ends. However, the functional relevance of such interactions and the molecular players are still under debate. Moreover, the involvement of these particular connections for the homeostasis of specific tissues remains unexplored.

In this thesis project we investigated how microtubules reach Adherens Junctions in basal cells of the epidermis. We identified a novel interaction between the microtubule plus-end binding protein CLASP2 and the cadherin binding molecule p120 at Adherens Junctions. In absence of CLASP2, Adherens Junctions do not form properly and present altered dynamics, which leads to a disruption of cell-cell adhesion. This phenotype is accompanied by alterations in microtubule dynamics at Adherens Junctions, in terms of speed and directionality. The same alterations are observed in absence of p120, suggesting that the CLASP2-p120 interaction contributes to microtubule targeting to Adherens Junctions.

Importantly, since CLASP2 expression is enriched in basal progenitor cells of the epidermis, the CLASP2-p120 interaction seems to be restricted to this specific epidermal compartment, where it may dynamically regulate cell-cell adhesion. On the contrary, the microtubule minus-end binding protein Nezha localizes to cell-cell contacts in suprabasal differentiated keratinocytes. Loss of CLASP2 leads to alterations in the proliferation-differentiation balance of basal keratinocytes. This alteration could be the result of weakened Adherens Junctions but it may also reflect a defect in the correct orientation of the spindle via interaction with p120.

Overall, the work presented in this doctoral thesis supports a model where microtubule plus-ends are targeted to Adherens Junctions in basal keratinocytes via the CLASP2-p120 interaction whereas minus ends are captured at cell-cell adhesion sites in differentiated cells. This could be an important mechanism to control Adherens Junctions stability and dynamics in epidermal progenitor cells.

Resumen

Las Uniones Adherentes son estructuras que contienen proteínas trasmembrana denominadas cadherinas, y desempeñan un papel fundamental para el mantenimiento de la adhesión celular, y por lo tanto, para preservar la correcta arquitectura y funcionalidad de un tejido. Para desempeñar esta función, las Uniones Adherentes establecen conexiones con el citoesqueleto celular, tanto con los filamentos de actina como con los microtúbulos. De esta manera, las células responden al daño o a los estímulos externos como parte integral de un tejido que funciona coordinadamente. Anteriores descubrimientos han demostrado la existencia de conexiones entre las cadherinas y los extremos positivos y negativos de los microtúbulos. Sin embargo, la relevancia funcional de estas interacciones, así como las moléculas implicadas, aún no han sido bien caracterizadas.

En esta tesis hemos investigado cómo los microtúbulos interaccionan con las Uniones Adherentes en las células basales de epidermis. Hemos identificado una nueva interacción entra la proteína de unión a los extremos positivos de los microtúbulos, CLASP2, y la proteína de unión a las cadherinas, p120, en las Uniones Adherentes. En ausencia de CLASP2, las Uniones Adherentes no se forman correctamente y presentan alteraciones en su dinámica, lo cual conduce a una pérdida de la adhesión celular. Asimismo, hemos detectado alteraciones en la dinámica de los microtúbulos en las Uniones Adherentes, en términos de velocidad y direccionalidad. Estas mismas alteraciones se observan en ausencia de p120, lo cual sugiere que la interacción entre CLASP2 y p120 contribuye a la correcta asociación de los microtúbulos con las Uniones Adherentes. *In vivo*, la expresión de CLASP2 está enriquecida en las células basales progenitoras de la epidermis. Por lo tanto, la interacción entre CLASP2 y p120 probablemente regula la dinámica de adhesión celular específicamente en este tipo de células epiteliales. Por el contrario, la proteína de unión a los extremos negativos de los microtúbulos, Nezha, se localiza en las zonas de unión célula-célula de los queratinocitos suprabasales. La pérdida de CLASP2 conduce a alteraciones en el balance de proliferación y diferenciación de los queratinocitos progenitores. Dicha alteración podría ser consecuencia del debilitamiento de las uniones celulares, pero puede también ser reflejo de un defecto en la correcta orientación del huso mitótico a través de la interacción con p120.

En resumen, el trabajo presentado en esta tesis apoya un modelo en el cual los extremos positivos de los microtúbulos se asocian con las Uniones Adherentes en los queratinocitos basales a través de la interacción entre las proteínas CLASP2 y p120. Por el contrario, los extremos negativos de los microtúbulos se asocian con las uniones célula-célula en queratinocitos diferenciados suprabasales. Esta interacción podría representar un mecanimo importante para controlar la estabilidad y la dinámica de las Uniones Adherentes en células progenitoras de la epidermis.

Table of contents

Abbreviations

αctn	α-catenin
ace-tub	Acetylated tubulin
AJs	Adherens Junctions
bio	Biotin
bp	Base pairs
CBD	Catenin Binding Domain
cfu	Colony-forming unit
DIC	Differential Interference Contrast Microscopy
EBH	End Binding Homology domain
ECad	E-Cadherin
ECM	Extracellular matrix
EMT	Epithelial to Mesenchymal Transition
ER	Endoplasmic Reticulum
ES cell	Embryonic Stem Cell
f/f	flox/flox
FAs	Focal Adhesions
FLP	Flippase
FRAP	Fluorescence Recovery After Photobleaching
FRT	Flippase Recognition Target
GAPs	GTPase-Activating Proteins
GEFs	Guanine nucleotide Exchange Factors
GJs	Gap Junctions
Glu	Detyrosinated tubulin
hKer	Human keratinocytes
HDAC	Histone DeACetylase
IP	Immunoprecipitation
JMD	Juxtamembrane Domain
K1	Keratin 1
K5	Keratin 5
K10	Keratin 10

Kbp	Kilo basepairs
LC	Low Calcium
LIF	Leukemia Inhibitory Factor
mAb	Monoclonal antibody
MAFs	Mouse Adult Fibroblasts
MAPs	Microtubule-associated proteins
MEFs	Mouse Embryonic Fibroblasts
mKer	Mouse keratinocytes
MSB	Microtubule Stabilizing Buffer
MTs	Microtubules
MTOC	Microtubule Organizing Centre
NC	Normal Calcium
NCad	N-Cadherin
pAb	Polyclonal antibody
PCad	P-Cadherin
pp	Primer pair
PS	PreScission Protease
ROI	Region Of Interest
RT-PCR	Real Time PCR
SB	Southern Blot
SCC	Squamous Cell Carcinoma
TJs	Tight Junctions
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labelling
Tyr	Tyrosinated tubulin
VECad	VE-Cadherin
WB	Western Blot
+TIPs	Microtubule Plus-End tracking Protein

Introduction

1. Epithelial architecture

1.1. An evolutionary perspective

During the course of evolution, the appearance of mechanisms of cell-cell adhesion has been instrumental for the origin of multicellular organisms. Multicellularity was acquired independently in the different multicellular kingdoms (animals, fungi and plants). Thus, each lineage evolved different cell-cell adhesion mechanisms (Abedin and King, 2010), which is reflected in the diversity of body plans observed in these multicellular kingdoms. In the case of the animal kingdom, the diverse mechanisms of cell adhesion allowed the formation of complex tissues with defined architectures and functions.

Peculiar of eumetazoans is the presence of the epithelium, a specialized tissue involved in secretion, absorption, protection, transport of solutes and detection of stimuli. Epithelia are characterized by the presence of cells with an aligned polarity, with a free apical domain, facing the lumen or the external environment, and a basal surface that interacts with the underlying basement membrane. Epithelial cells are held together by cell-cell junctions (Tyler, 2003). These junctions fulfill multiple functions including protection against mechanical stress, communication between cells and formation of a barrier. Epithelial tissues in vertebrates show four major types of cell-cell junctional complexes: Adherens Junctions (AJs), Tight Junctions (TJs), desmosomes and Gap Junctions (GJs). But, which molecules mediated cell-cell adhesion in the early metazoan ancestor?

The first molecules known to mediate cell-cell adhesion during the evolution of the animal kingdom are classical cadherins (those found in AJs). Classical cadherins are present in all metazoans, ranging from sponges to humans (Abedin and King, 2010). Moreover, in lower organisms such as choanoflagellates (unicellular and colonial organisms considered to be the closest living relative of animals) there are 23 putative cadherin genes, although their functional role is currently unknown (Abedin and King, 2008). Homologous proteins of catenins, which are part of AJs, have also been identified in the non-metazoan *Dictyostelium discoideum* (Dickinson et al.; Grimson et al., 2000). These findings suggest that the origin of AJs proteins predates the origin of metazoans, and these proteins were co-opted later during evolution to mediate cell-cell adhesion.

Given that ontogeny recapitulates phylogeny, in *Drosophila*, *Caenorhabditis* and vertebrates, the first indication of epithelial characteristics in the embryonic blastomeres is the formation of cadherin-based adhesions (Tyler, 2003). Thus, AJs associated proteins not only played a major role in the generation of multicellular epithelia during evolution, but they are also fundamental for morphogenesis. TJs and GJs appeared later during evolution and perform a barrier function and

allow communication between cells, respectively. The desmosome was a final elaboration of vertebrate tissues that resists mechanical stress (Abedin and King, 2010). Different combinations of junctional complexes are observed in different epithelial tissues, leading to a great variety of architectural and functional features.

1.2. Cell adhesion: molecular basis of epithelial architecture

Tissue architecture is based upon proper cell-cell and cell-matrix interactions (Hagios et al., 1998), which generate defined tridimensional structures and participate in the regulation of tissue function (Gumbiner, 1996). In general terms, junctional complexes are composed of a transmembrane adhesion receptor, with an extracellular domain responsible for establishing either cell-cell or cell-matrix interactions. The intracellular domain of the adhesion receptor interacts with cytoplasmic proteins that generate a link with the cytoskeleton and dynamically regulate cell adhesion, or other aspects of cell behaviour (Gumbiner, 1996).

1.2.1. Cell – Extracellular Matrix Interactions

The paradigm of cell – extracellular matrix (ECM) interactions is represented by integrin receptors. Integrins are heterotrimer receptors composed of α and β subunits that interact with components of the ECM, such as collagen fibers and fibronectin molecules (Campbell and Humphries, 2011). Integrins are found in two different kinds of junctional complexes: focal adhesions (FAs), which link the actin cytoskeleton to the integrin receptor and are involved in cell attachment, cell migration and signaling (Lo, 2006); and hemidesmosomes, which represent attachment sites for intermediate filaments, and thus generate resistance to mechanical stress (Green and Jones, 1996).

1.2.2. Cell- cell interactions

Cell-cell junctions were first described in 1963 by Marilyn Farquhar and George Palade using electron microscopy (Farquhar and Palade, 1963). The latter was awarded the Nobel Prize in 1974 for “his discovery concerning the structural and functional organization of the cell”. The authors already noted the polarized distribution of junctional complexes in epithelial tissues, which they termed “the Tripartite Junctional Complex”: TJs, AJs and desmosomes.

- Tight Junctions: TJs are the most apical structure of the Tripartite Junctional Complex and appose membranes from neighboring cells virtually obliterating the extracellular space between them. These two architectural features dictate the two major functions of TJs in epithelia: they form a permeable barrier to control the passage of solutes through the paracellular space, and they generate two functionally and biochemically distinct domains within the epithelium: the apical domain and the basolateral domain (Gumbiner, 1996; Niessen, 2007). TJs are composed by transmembrane occludins, claudins, and junctional adhesion proteins (JAMs), which directly interact with a scaffold plaque of cytosolic proteins composed mainly of PDZ-domain containing

proteins ZO-1, ZO-2 and ZO-3. These proteins establish a link with the actin cytoskeleton (Niessen, 2007) and are closely associated to apical polarity proteins, therefore affecting the establishment and maintenance of apico-basal polarity (Martin-Belmonte and Perez-Moreno, 2012) (Fig. 1).

- Adherens Junctions: AJs are localized below TJs and are visualized by electron microscopy as defined membrane structures in which cells are separated by an extracellular space of approximately 20 nm (Farquhar and Palade, 1963). The main function of AJs is to maintain the physical association between cells (Meng and Takeichi, 2009). AJs are mainly composed of cadherin adhesion receptors that mediate homotypic interactions and are organized into clusters of different sizes, generating a modular organization of the junction (Harris and Tepass, 2010). Cadherins interact intracellularly with a group of proteins termed catenins. They were initially discovered in 1989 as cadherin-binding proteins and termed catenins (*catena*, the Latin name for chain) since they were thought to mediate connections between AJs and the cytoskeleton (Ozawa et al., 1989). p120-catenin (p120) binds directly to the juxtamembrane domain (JMD) of the cadherin tail and controls its stability at the plasma membrane (Davis et al., 2003). β-catenin is well-known for its signaling function in the Wnt pathway as a transcriptional coactivator, but it also binds the C-terminal domain of cadherins, the so called catenin-binding domain (CBD), mediating the connection with α-catenin (Perez-Moreno et al., 2003). In turn, α-catenin interacts with actin binding proteins connecting the cadherin complex to the actin cytoskeleton (Harris and Tepass, 2010) (Fig. 1).

Cadherin deficiency in various cell lines as well as in primary mouse keratinocytes (mKer) leads to alterations in the formation of desmosomes, TJs and GJs (Gumbiner et al., 1988; Michels et al., 2009; Musil et al., 1990; Watabe et al., 1994), supporting a major role for AJs in cell-cell adhesion. *In vivo* loss-of-function models in the epidermis confirm the requirement of AJs for the formation of TJs (Tinkle et al., 2008).

AJs also contain IgG-like nectin adhesion receptors that mediate both homotypic and heterotypic interactions. They interact intracellularly with the actin binding protein afadin, thus connecting the junction to the actin cytoskeleton (Niessen, 2007). Afadin is in turn able to interact with α-catenin, providing a link between cadherin and nectin adhesion receptors at AJs (Tachibana et al., 2000). This link may be involved in the initial formation of contacts, since nectin-afadin seems to be required to prime the formation of cadherin-based adhesions (Takai et al., 2008).

- Desmosomes: Desmosomes preserve tissue architecture in situations of strong physical stress, for example in the cardiac muscle or the epidermis. As a matter of fact, several autoimmune blistering diseases are a consequence of desmosomal alterations (Green and Simpson, 2007). Desmosomes were first described as two dense cytoplasmic plaques that leave a space of approximately 40 nm between adjacent cells and are connected to fibrils on the cytosol. In simple epithelia, desmosomes

localize basal to the AJs (Farquhar and Palade, 1963). Nowadays, we know that desmosomes are composed of transmembrane proteins of the cadherin family, the so-called desmosomal cadherins. These cadherins interact intracellularly with plakoglobin and plakophilins, which are homologous to β-catenin and p120-catenin respectively, and connect the desmosomal plaque to intermediate filaments through interactions with desmoplakin. The result is the formation of a network of intermediate filaments throughout the tissue, which is connected to both desmosomes and hemidesmosomes (Green and Jones, 1996). More recently, signaling functions have been attributed to desmosomes. In particular, desmosomal cadherins seem to control mKer differentiation in the epidermis (Simpson et al., 2011) (Fig. 1)

- Gap Junctions: GJs serve the purpose of communication between cells: they are composed of clusters of channels made by the oligomerization of connexins that link the cytoplasm of two cells and allow the exchange of ions, small metabolites and second messengers, among others. As a result, cells within a tissue can be physiologically synchronized (Mese et al., 2007). GJs are not exclusive of epithelial tissues but play fundamental roles in the liver, nervous system, heart, muscle, etc (Mese et al., 2007). Not surprisingly, various human diseases have been associated to mutations in connexin proteins (Richard, 2000) (Fig. 1).

Given the important contributions that AJs make to the formation of the "Tripartite Junctional Complex" during embryogenesis and tissue homeostasis, we are going to focus on these structures with more detail in the following sections.

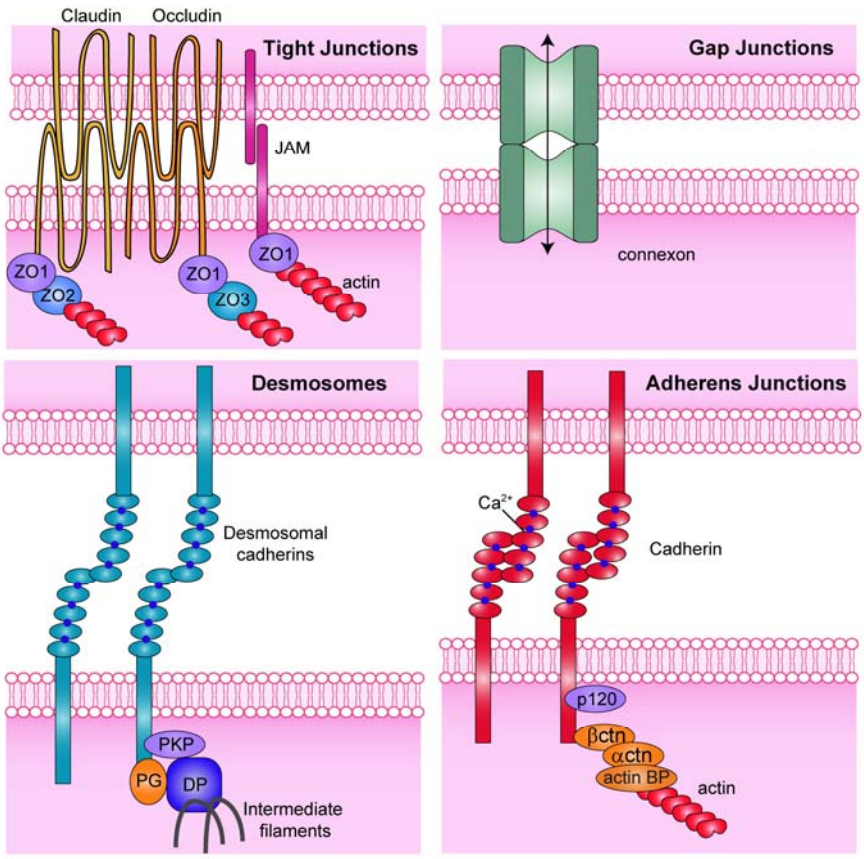


Figure 1 Schematic representation of epithelial cell-cell junctions. Epithelial tissues present four major types of cell-cell junctions: Tight Junctions, Adherens Junctions, Gap Junctions and desmosomes. They share a common structure consisting of a transmembrane adhesion receptor that through its extracellular domain mediates binding to other receptors on the surface of neighboring cells. The intracellular domain of the adhesion receptor interacts with cytoplasmic proteins that generate a link with the cytoskeleton and modulate cell-cell adhesion.

2. Adherens Junctions

AJs are implicated in morphogenetic movements and the formation of cohesive interactions between cells (Gumbiner, 2005). They have been also implicated in other aspects beyond their role in intercellular adhesion. For example, AJs maintain tissue homeostasis by coordinating growth and differentiation in the gut (Hermiston et al., 1996) and the epidermis (Tinkle et al., 2004), regulating the passage of solutes in endothelial tissues (Dejana et al., 2009), or regulating neuronal synapses in the brain (Togashi et al., 2002). As mentioned before, AJs are composed of two main adhesive modules: cadherin-catenins and nectins-afadin. The following description will focus only on the cadherin-catenin complex since its roles in the establishment and maintenance of tissue architecture are better defined (Niessen et al., 2011).

2.1. Cadherins

At the beginning of the 20th century, embryologists reported a phenomenon of cell-cell aggregation mediated by calcium during the development of organisms as diverse as sponges and amphibians (Franke, 2009). It was not until the late 70s when the groups led by Takeichi, Kemler and Jacob identified the responsible molecule, which was named uvomorulin (due to its role in cell compactation at the morula stage), or alternatively cadherin (due to its dependence on extracellular calcium) (Hyafil et al., 1981; Takeichi, 1977; Vestweber and Kemler, 1984). The first component of the AJs had been identified.

Nowadays, cadherins constitute a superfamily of proteins with roles beyond cell-cell adhesion. The superfamily is defined based on the presence of the so called Extracellular Cadherin repeats (EC repeats), characterized by their binding to calcium (Niessen et al., 2011). Cadherins can be classified into several subfamilies according to their structure and function (Halbleib and Nelson, 2006). While classical cadherins are involved in homotypic cell-cell interactions within the AJs, non-classical cadherins are not well understood with respect to their adhesive mechanisms. Structurally, classical cadherins are single-pass transmembrane glycoproteins characterized by the presence of five extracellular calcium binding EC repeats and a conserved intracellular domain that binds to β-catenin and p120 (Niessen et al., 2011).

The different members of the subfamily were initially named after the tissue in which they are preferentially expressed (e.g. E, epithelial, N, neural). Although the sequences of the EC repeats are highly conserved, changes of other sequences in the extracellular domain mediate the specificity of each member. This particular feature underlies the cell sorting phenomenon observed when different classical cadherins are expressed in a population of cells: for example, cells expressing ECad will interact with cells expressing also ECad and not with cells expressing other cadherin type (Meng and Takeichi, 2009). In addition, EC repeats strengthen the binding by mediating not only

trans-interactions between cadherins on opposing membranes, but also cis-interactions, leading to the formation of cadherin dimers, oligomers and clusters. The formation of such clusters is controlled by the binding of intracellular molecules to the cadherin tail and directly modulates the strength of cell-cell adhesion (Niessen et al., 2011).

The dynamic regulation of cadherin cell adhesion and its specific spatio-temporal expression pattern is critical during development and adult tissue homeostasis. At the organismal level, absence of ECad is embryonically lethal at the blastocyst stage (Larue et al., 1994). In adult tissues, the relevance of cadherin-mediated adhesion is best exemplified by its role in cancer progression and metastasis. Loss of ECad expression, together with upregulation of N-Cadherin (NCad), is associated with loss of epithelial characteristics and increased cell migration: a process known as epithelial to mesenchymal transition (EMT) (Hanahan and Weinberg, 2011). Further, it has been demonstrated a causal role for ECad deficiency in the progression of adenomas to more malignant carcinomas in a mouse model of pancreatic carcinogenesis (Perl et al., 1998).

Due to its relevance in human disease, substantial efforts have been made to understand the mechanisms that regulate the surface levels of cadherins. Phosphorylation, proteolytic cleavage, regulation of actomyosin contractility and trafficking of cadherins are all dynamic processes that impact on cadherin levels at the membrane, and thus, on the adhesive properties of cells (Niessen, 2007). Cadherins are also regulated at the transcriptional level. It is well established that ECad is silenced in mesenchymal cells and during EMT (Cano et al., 2000; Jamora et al., 2003; Ocana et al., 2012; Peinado et al., 2004; Perez-Moreno et al., 2001). However, there are still open questions about the dynamic regulation of cadherin levels and its turnover at the membrane. The role of cytoskeletal interactions, particularly with MTs and their associated proteins, is still not well understood. These processes will be explored with more detail in the next chapters.

2.2. Catenins

Although catenins were originally described as molecules found in cell-cell adhesion complexes, nowadays it is clear that they can play many different roles at diverse subcellular locations (Perez-Moreno and Fuchs, 2006).

2.2.1. β-catenin

β-catenin is characterized by the presence of the so-called Armadillo repeats, helical domains that usually appear in tandem. Specifically, β-catenin presents 12 armadillo repeats that mediate the binding to cadherins. Binding to α-catenin takes place at the N-terminal region (Shapiro and Weis, 2009). β-catenin is known for its dual role both at AJs (bridging α-catenin and actin filaments to cadherins), and at the nucleus as a transcriptional co-activator in the Wnt/β-catenin signaling pathway, with major implications for cell fate and tissue renewal (Bienz, 2005).

2.2.2. α-catenin

α-catenin lacks armadillo domains; instead, it is composed of an N-terminal homodimerization domain that overlaps with the β-catenin binding domain, a middle M domain that binds afadin, and a C-terminal actin binding domain (Shapiro and Weis, 2009). Homodimerization of α-catenin and binding to β-catenin are mutually exclusive events. This is of relevance for the architecture of AJs, since the α-catenin dimer binds to actin more strongly than the monomer. As a matter of fact, monomeric α-catenin mediates the anchorage of actin filaments to the junction indirectly, via intermediate actin binding proteins such as vinculin, α-actinin, formin or eplin (Drees et al., 2005). Thus, α-catenin plays a fundamental role for the maintenance of cell-cell adhesion by stabilizing junctions and integrating actin networks across epithelial tissues (Perez-Moreno and Fuchs, 2006). Such a fundamental role is revealed in an α-catenin loss-of-function mouse model in which development is blocked at the blastocyst stage (Torres et al., 1997), mimicking the phenotype of ECad deficient embryos (Larue et al., 1994). Additionally, non-canonical roles for α-catenin have been described such as spindle positioning (Lechler and Fuchs, 2005) or regulation of microtubule (MT) stability (Shtutman et al., 2008).

2.2.3. p120-catenin

p120 was discovered as a Src substrate (Reynolds et al., 1989) and later it was shown to directly bind cadherins through its Armadillo domain (Ishiyama et al., 2010; Reynolds et al., 1994). Nowadays, different members of a p120-catenin subfamily have been described in vertebrates, including: p120, Armadillo repeat gene deleted in Velo-Cardio-Facial syndrome (ARVCF), δ-catenin and p0071. Intriguingly, Drosophila possesses one single p120 protein that does not play a major role in cell-cell adhesion (McCrea and Park, 2007). We will focus on the most abundant member of the family, p120.

p120 contains a central armadillo domain with 9 armadillo repeats (Shapiro and Weis, 2009). The N-terminal domain has mainly a regulatory function via Ser and Tyr phosphorylation. It varies in length due to the presence of four transcription starting sites which give rise to tissue-specific isoforms. In broad terms, isoform 1 is characteristic of fibroblast-like cells, whereas epithelial cells preferentially express isoform 3 (Anastasiadis and Reynolds, 2000).

The following adhesion and non-adhesion functions have been attributed to p120:

- Role of p120 in cadherin trafficking: p120 is best known for regulating cadherin stability at the membrane. In absence of p120 cadherins are rapidly internalized and degraded (Davis et al., 2003; Ireton et al., 2002; Reynolds and Carnahan, 2004) (Fig. 2).

The life of a cadherin molecule begins upon translation in the endoplasmic reticulum (ER) following processing in the Golgi. It has been shown that β-catenin associates to the cadherin tail at the ER and both proteins are transported to the membrane together (Hinck et al., 1994). However, the

involvement of p120 in this process is still controversial. One line of evidence suggests that p120 is incorporated into the cadherin complex once cadherins have reached the membrane, and thus p120 would not be required for cadherin exocytosis (Davis et al., 2003; Miranda et al., 2003). On the contrary, work from the lab of M. Wheelock suggests that p120 interacts with cadherin precursors (both pro-ECad and pro-NCad) at the ER (Curtis et al., 2008; Wahl et al., 2003). In particular, NCad containing vesicles move using MT tracks (Mary et al., 2002) in a kinesin and p120-dependent manner (Chen et al., 2003). p120 has been shown to directly interact with MTs and kinesin motors (Franz and Ridley, 2004; Yanagisawa et al., 2004), raising the possibility that a kinesin-p120-NCad complex mediates the arrival of newly synthesized cadherin molecules to the membrane (Peifer and Yap, 2003).

Regardless of the initial trafficking to the membrane, it is well established that once cadherins reach the surface, p120 binding to the JMD is necessary to masks an endocytic signal within the JMD. Release of p120 exposes this signal, induces cadherin internalization and degradation in lysosomes (Xiao et al., 2007). The nature of this endocytic signal is still unclear. The internalization of most classical cadherins may involve an endocytic dileucine motif (LL motif) that recruits clathrin adaptor complexes for the endocytosis of the cadherin (Ishiyama et al., 2010; Miyashita and Ozawa, 2007). Another pathway, may be the recognition of a Tyr phosphorylated residue of the JMD and subsequent ubiquitination of the cadherin by the E3 ubiquitin ligase Hakai (Fujita et al., 2002). However, this Tyr residue is only present in ECad. Yet another possibility may be the cleavage of cadherins mediated by the γ-secretase presenilin-1, which has been shown to compete with p120 for cadherin binding (Baki et al., 2001; Marambaud et al., 2002). A recent report has identified a specific p120-binding domain that masks an endocytic signal conserved in classical cadherins (Nanes et al., 2012). The common feature in all these cases is the protective function of p120. But, how does p120 dynamically disassociate from the cadherin tail? This is one of the most critical questions that remain to be addressed in order to understand how p120 controls cadherin stability at the membrane (Nanes et al., 2012; Xiao et al., 2007).

Internalized cadherins can be either recycled back to the membrane or delivered to lysosomes for degradation. The molecular signaling pathways leading to one or the other destination need to be further explored (Bryant and Stow, 2004). What is clear nowadays is that static AJs cannot fulfill their major role as morphogenetic regulators; instead, dynamic cadherin recycling is fundamental for processes such as cell migration and tissue patterning (Nanes et al., 2012).

- <u>Role of p120 as a master regulator of RhoGTPases</u>: RhoGTPases are molecular switches that can be in an active state (bound to GTP) or in an inactive state (bound to GDP). When active, RhoGTPases interact with multiple effector proteins, including major cytoskeletal regulators. Guanine nucleotide exchange factors (GEFs) catalyze GDP exchange for GTP leading to activation of RhoGTPase,

whereas GTPase-activating proteins (GAPs) stimulate GTP hydrolysis leading to their inactivation (Etienne-Manneville and Hall, 2002). The three best characterized members of the family are: Rho, Rac and Cdc42. Rho is best known for regulating the actin cytoskeleton, inducing acto-myosin contractility and formation of stress fibers, yet it also induces MT stabilization during cell migration (Palazzo et al., 2001). Rac mediates actin polymerization and lamellipodia formation, playing a fundamental role during cell migration and cell-cell contact formation. Cdc42 induces formation of actin filaments in the form of filopodia and is a major polarity regulator (Schwartz, 2004).

p120 inhibits RhoA activity by inhibiting the exchange of GDP for GTP (Anastasiadis et al., 2000). It also activates both Rac and Cdc42, which could lead to further RhoA inactivation (Anastasiadis, 2007). These activities can take place in the cytoplasm in a cadherin-independent fashion but they have also been reported at cell-cell contacts and have implications not only for contact formation, but also for cell migration or cytokinesis (Anastasiadis, 2007) (Fig. 2).

- Role of p120 as a transcriptional coactivator: p120 has been observed in the nucleus of various human cell lines and tumor tissues (Roczniak-Ferguson and Reynolds, 2003). This localization seems to be functionally relevant, since p120 directly interacts with the transcription factor Kaiso, repressing its activity. Kaiso is a zinc finger protein that belongs to the BTB/POZ subfamily of transcription factors and recruits histone deacetylases (HDACs) leading to gene silencing (Daniel, 2007; Daniel and Reynolds, 1999). Especially noteworthy is the regulation by Kaiso/p120 of Wnt/β-catenin target genes such as cyclinD1, matrilysin or siamois, creating a cross-talk between the signaling pathways mediated by both catenins (Daniel, 2007) (Fig. 2).

- p120 and the microtubule network: p120 has been shown to localize to the centrosome in human carcinoma cell lines and regulate CDK2/cycE complexes. In this regard, increasing levels of p120 are associated to defects in centrosome separation and chromosome instability (Chartier et al., 2007). Various studies also report p120 colocalization with the MT network, mitotic spindles and kinesin motors (Franz and Ridley, 2004; Roczniak-Ferguson and Reynolds, 2003; Yanagisawa et al., 2004) (Fig.2). As mentioned previously, these interactions could be relevant for cadherin delivery to the membrane (Chen et al., 2003; Mary et al., 2002), although they could also play cadherin-independent functions, since it has been described that p120 stabilizes MTs in a cadherin-independent manner (Ichii and Takeichi, 2007). The relevance of p120 interactions with MTs in the dynamic regulation of AJs will be discussed in more detail in the following sections.

2.3. Formation and maintenance of AJs

We have described how the different components of AJs interact with each other and with elements of the cytoskeleton, and how in some cases they signal inside the cell. But, how all these

pieces of the puzzle are put together to form and maintain mature AJs? AJs formation takes place at exploratory membrane protrusions (lamellipodia) where nectin-afadin complexes cluster (Harris and Tepass, 2010; Martin-Belmonte and Perez-Moreno, 2012). Afadin recruits both polarity proteins and cadherin-catenin proteins that form the initial nascent cell-cell contact at filopodia in a calcium-dependent manner (Vasioukhin et al., 2000). Cadherins in turn can recruit TJs components as well as activate RhoGTPases (Perez-Moreno et al., 2003). In the initial stages of contact formation, Rac and Cdc42 play a major role. Rac induces actin polymerization and maturation of the initial cadherin contacts, whereas Cdc42 activates the polarity proteins leading to the separation of TJs and AJs into discrete functional domains (Perez-Moreno and Fuchs, 2006). At later time points, the activation of RhoA is crucial to generate tension via actomyosin contractility and to promote more robust cell-cell interactions (Niessen et al., 2011). Importantly, the crosstalk between AJs and RhoGTPases is bidirectional: RhoGTPases participate in the formation and maintenance of AJs, whereas AJs modify the activity of these GTPases leading to changes in the structure and polarity of the cell (Baum and Georgiou, 2011). Once formed, AJs must be able to resist forces that otherwise would break cell-cell contacts as well as to transmit forces to adjacent cells. This is mainly achieved through the connections between cadherin-catenins and the actin cytoskeleton as previously described. MTs have also been observed at AJs (Akhmanova et al., 2009; Stehbens et al., 2006). But, how do they interact with junctional components? Which molecules are implicated in the link between AJs and MTs? And what role do they play during the formation and maintenance of AJs? Before addressing the key issues related to these questions, we will describe the characteristics of MTs with more detail in the following section.

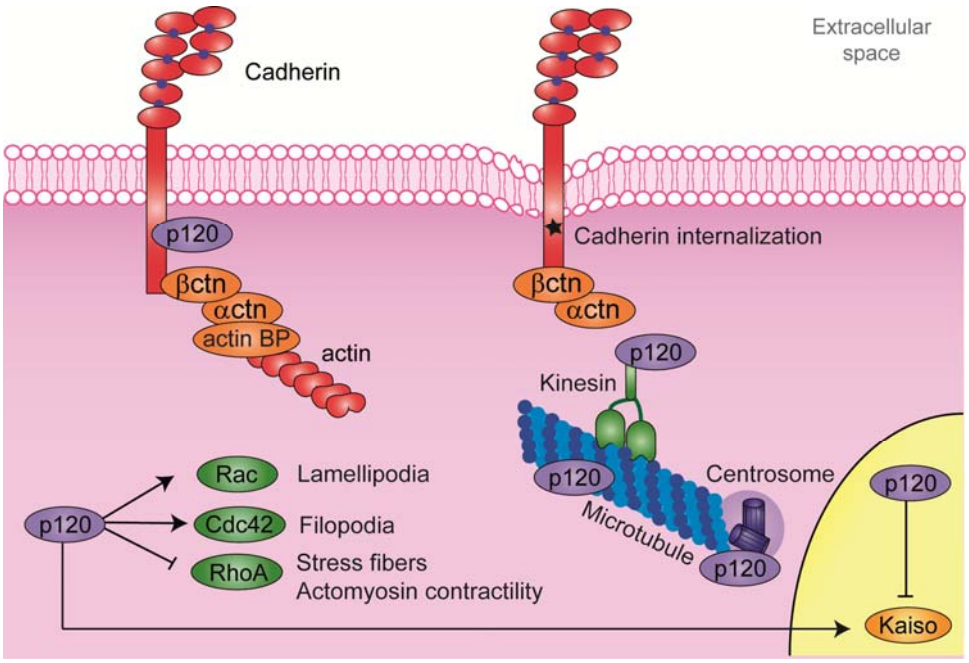


Figure 2 Adhesive and non-adhesive functions of p120. p120 is found in different subcellular localizations. At the membrane it controls cadherin stability, in the cytosol regulates the RhoGTPases, binds microtubules, centrosomes and kinesins, and in the nucleus controls the activity of Kaiso.

3. Microtubules and Adherens Junctions

3.1. General features of microtubules

MTs together with actin and intermediate filaments form the cytoskeleton. MTs are required for cell division, motility, vesicular traffic and polarized localization of organelles in all eukaryotic cells (Brinkley, 1997). One of the most remarkable properties of MTs is their ability to generate different types of networks such as the mitotic spindle in dividing cells, radial MT arrays (fibroblasts), parallel arrays of MTs directed to the cell cortex (polarized epithelial cells), or highly stable structures such as centrioles, flagella and cilia of cells in interphase.

In the late 60s, tubulin was identified as the basic subunit of MTs (Borisy and Taylor, 1967). Tubulin is a heterodimer of α and β subunits that interact non-covalently to generate the functional unit of the protein. Tubulin heterodimers bind in a polar fashion to form protofilaments. The arrangement of 13 protofilaments into a 25 nm hollow cylindrical structure generates the MT. As a consequence of the polarized addition of tubulin heterodimers, MTs have a characteristic polarity with a minus-end (MT end with α-tubulin) and a plus-end (MT end with β-tubulin) (Nogales, 2000). *In vitro*, plus-ends are very dynamic and fast growing, whereas minus-ends grow more slowly and are less dynamic (Summers and Kirschner, 1979).

The expression of tissue specific α and β tubulin isotypes generates MT diversity. This diversity is greatly increased by a number of post-translational modifications, namely: acetylation, detyrosination, polyglutamylation and polyglycylation (Westermann and Weber, 2003).

These post-translational modifications are the result of changes in MT dynamics (Bulinski and Gundersen, 1991) and need to be controlled for the correct functioning of cells (Erck et al., 2005; Peris et al., 2006). At any given time, MTs can be growing (by addition of tubulin dimers), shrinking (by depolymerization of MT subunits) or pausing (no net changes in the length of the MT). The combination of growth, shrinkage and pause is termed dynamic instability (Burbank and Mitchison, 2006). A transition from a growing phase to a shrinking phase is called catastrophe, whereas the opposite process is termed rescue (Akhmanova and Steinmetz, 2008). Dynamic instability allows the cell to rapidly respond to environmental cues, reorganizing the cytoskeleton if necessary (Burbank and Mitchison, 2006). As an example of this remarkable dynamicity, the half life of a MT in a fibroblast in culture is 5 minutes (Schulze and Kirschner, 1986).

3.2. Microtubule organization

MTs need to be organized in order to be functional. How is the MT network organized in a cell? In 1969 the term Microtubule Organizing Centre (MTOC) was coined by Pickett-Heaps and defined as a structure from which MTs were assembled and organized (Luders and Stearns, 2007). Classically, the centrosome is considered to be the MTOC in animal cells. However, now we know that MTs can be polymerized and organized from different structures within cells.

3.2.1. Centrosomal microtubule organization

Centrosomes were initially described by Theodor Boveri at the beginning of the 20th century as 1 µm non-membrane bound organelles. They are composed of two centrioles surrounded by a pericentriolar material, which contains numerous proteins necessary for MT nucleation (γ-tubulin) and MT anchoring (e.g. ninein or dynein) (Kitagawa et al., 2011; Schatten, 2008). Typically, MTs are organized with their minus-ends located at the centrosome and their dynamic plus-ends exploring the periphery of the cell. γ-tubulin together with a number of associated proteins, forms the highly conserved γ-tubulin ring complex. This complex is implicated in the nucleation of new MTs and the capping and stabilization of MT minus-ends to prevent their depolymerization (Dammermann et al., 2003). A centrosomal MT organization generates a radial MT array (Keating and Borisy, 1999).

3.2.2. Non-centrosomal microtubule organization

Non-centrosomal MTOCs were first described in plants (lacking centrosomes) and later found also in animal cells. They represent non-centrosomal accumulations of γ-tubulin capable of nucleating and stabilizing MTs (Luders and Stearns, 2007). A non-centrosomal MT organization typically generates a linear MT array such as those observed in polarized epithelial cells, myotubes or neurons (Bartolini and Gundersen, 2006; Musch, 2004; Stiess et al., 2010). It was initially thought that non-centrosomal MTs were characteristic of differentiated cells that needed to adapt their morphology to a very well defined polarity. However, non-centrosomal MT arrays have been described in non-polarized undifferentiated cells (Efimov et al., 2007). Depending on the cellular system, MTs minus-ends can be organized from the Golgi (Efimov et al., 2007), the nuclear envelope in myotubes (Bugnard et al., 2005), the plasma membrane of polarized epithelial cells (Meng et al., 2008; Reilein and Nelson, 2005) or melanosomes of pigmented cells (Malikov et al., 2004).

3.3. Microtubule-associated proteins

Microtubule-associated proteins (MAPs) were initially defined as proteins that co-purify with tubulin. Nowadays the definition includes all proteins that directly bind to tubulin or to MTs, either *in vitro* or *in vivo* (Gouveia and Akhmanova, 2010). The major role of MT binding proteins is to regulate MT dynamics. MAPs can be classified according to the way they bind MTs and their effect on MTs into: classical MAPs, MT motors and MT plus-end tracking proteins (+TIPs). Additional MT binding proteins include MAPs that nucleate MTs, such as γ-tubulin; or MAPs that destabilize MTs, such as katanin or stathmin (Amos and Schlieper, 2005).

Classical MAPs are proteins that bind to the whole lattice of MTs. Generally, they present MT stabilizing effects and have been studied in the context of the highly stable MT networks of neurons due to its relevance for Alzheimer disease. This family includes the proteins MAP2 (concentrated in dendrites) and Tau (concentrated in axons) (Amos and Schlieper, 2005).

Regarding MT motors, there are two major families: kinesins and dynein. Both types use the energy of ATP hydrolysis to transport cargoes along MTs towards their final destinations with a high

processivity (Gennerich and Vale, 2009). Most kinesins "walk" on the surface of the MT transporting cargoes towards the plus-end (Caviston and Holzbaur, 2006); whereas dynein (together with its accessory complex dynactin) mediates minus-end directed transport of cargoes (Caviston and Holzbaur, 2006).

+TIP proteins are one of the most conserved components of the MT cytoskeleton, been present in all eukaryotes from yeasts to plants and animals (Schuyler and Pellman, 2001). As opposed to conventional MAPs, +TIPs track only the ends of MTs, which dictates their cellular functions. They are well known regulators of MT dynamics and mediate MT interactions with other cellular structures, such as cortical sites, kinetochores or vesicles (Akhmanova and Steinmetz, 2010). Some +TIP proteins interact with both actin and MTs, and thus can participate in the actin-MT crosstalk (Rodriguez et al., 2003). Moreover, many +TIPs are found at the centrosome, yet their functional relevance at this location is currently unknown (Jiang and Akhmanova, 2011). It is important to mention that +TIPs proteins have been proposed to bridge MTs with cortical sites leading to MT stabilization (Gundersen, 2002). A particular type of cortical-MT interactions is the anchorage of MTs at AJs and we will describe this process with more detail in the following sections.

3.4. Classification of +TIP proteins

Since the discovery of the first +TIP protein, termed cytoplasmic linker protein 170 (CLIP170), (Perez et al., 1999), more than 20 types of +TIP proteins have been identified. The most relevant ones are mentioned below.

3.4.1. EB family

End Binding (EB) proteins (EB1, EB2 and EB3 in mammals) are evolutionary conserved proteins considered to be the core element of +TIP networks: they are track MT plus-ends independently of other +TIPs, they directly associate with practically all +TIP proteins (Akhmanova and Steinmetz, 2010) and they are necessary for most +TIPs to track MT plus-ends (Kumar and Wittmann, 2012). EB proteins localize to growing MT plus-ends, centrosomes and the midbody. *In vivo*, their major role is to inhibit MT catastrophes and promote MT growth (Gouveia and Akhmanova, 2010).

3.4.2. CAP-Gly proteins

This family of +TIPs includes CLIP proteins (CLIP-170 and its brain-specific relative CLIP-115) and the subunit of the dynactin complex $p150^{glued}$. These proteins are characterized by the presence of the highly conserved cytoskeleton-associated protein-glycine rich (CAP-Gly) domain at their N-terminal region, which mediates the interaction with EB proteins, tubulin subunits and MTs (Gouveia and Akhmanova, 2010). Upon binding to MTs, CLIPs act as rescue factors in mammalian cells, preventing MT catastrophes and aiding in subunit addition to the MT plus-end (Slep and Vale, 2007).

3.4.3. Proteins with basic and Ser-rich regions

This family of proteins includes a diverse group of +TIPs characterized by the presence of unstructured regions enriched in serines and basic residues (Akhmanova and Steinmetz, 2008). These regions contain the phosphorylation controllable motif Ser/Thr-X-Ile-Pro (SxIP), which mediates MT plus-end targeting in an EB1-dependent manner (Galjart, 2010; Kumar and Wittmann, 2012). The main members of this family are APC, ACF7 and CLASPs.

- APC: Adenomatous polyposis coli (APC) is best known for its role as a tumor suppressor in the Wnt/β-catenin signaling pathway. Less recognized is its role as a MT plus-end binding protein that induces MT stabilization (Mimori-Kiyosue and Tsukita, 2001). Heterozygous mutations in APC lead to spindle orientation defects in the intestine, a step that may represent an early event during intestinal tumorigenesis (Quyn et al., 2010).
- ACF7: Actin crosslinking family 7 (ACF7) is a member of the spectraplakin family of proteins, characterized by its ability to bind both actin and MTs. Recent studies in mKer have shown that ACF7 has an ATPase activity, which is required for transporting +TIP complexes along actin filaments (Wu et al., 2008). Thus, ACF7 not only regulates MT dynamic instability as a +TIP protein (Kodama et al., 2003), but it also coordinates the polarized movement of MTs along actin filaments.
- CLASPs: Mammalian CLASPs (CLASP1 and CLASP2) were identified as CLIP-Associated Proteins (Akhmanova et al., 2001) and are conserved between yeasts, plants and animals. CLASPs contain a basic Ser-rich region with two typical SxIP motifs that mediate binding to MT plus-ends in an EB1 dependent manner. The C-terminal region mediates interactions with CLIPs, kinetochores, Golgi and cortical proteins such as LL5β; and the N-terminal region contains a TOG domain whose function is unknown (Galjart, 2005; Gouveia and Akhmanova, 2010).

In *Drosophila,* there is one CLASP homologous protein, Orbit/Mast, which plays an essential role during mitosis stabilizing MTs at kinetochores (Maiato et al., 2005; Maiato et al., 2002). The CLASP orthologs Xorbit in *Xenopus*, and cls-2, in *C.elegans*, have been shown to perform an equivalent function during meiosis and mitosis (Cheeseman et al., 2005; Hannak and Heald, 2006). In higher plants, *Arabidopsis* CLASP mediates MT-cortex interactions (Ambrose and Wasteneys, 2008). In mammals, CLASPs are major MT stabilizing factors at the cortex: they prevent catastrophe and promote pausing (Drabek et al., 2006; Mimori-Kiyosue et al., 2005). Most importantly, they do it in a polarized fashion: in migrating fibroblasts and human keratinocytes CLASPs decorate the distal segments of lamella MTs. This process is negatively regulated by GSK-3β (Kumar et al., 2009; Wittmann and Waterman-Storer, 2005). During mitosis, CLASPs are needed for normal MT-kinetochore attachments and the maintenance of spindle bipolarity (Logarinho et al., 2012; Maiato et al., 2003; Pereira et al., 2006). Moreover, CLASPs control MT nucleation and anchoring at the trans-Golgi (Efimov et al., 2007), as well as clustering of Golgi stacks after mitosis (Miller et al., 2009). Thus, CLASPs are stabilizing +TIP proteins that function locally, contributing to the polarization of cells during processes such as cell migration or vesicle trafficking.

CLASP1 and CLASP2 present very similar biological functions. The development of a CLASP2 KO mouse model allowed the identification of CLASP2-specific functions at the organism level (Drabek et al., 2006). CLASP2 plays a fundamental role to capture MTs at postsynaptic structures of the neuromuscular junction (Schmidt et al., 2012) and is required for hematopoietic stem cell (SC) maintenance (Drabek et al., 2012). These recent findings place CLASPs as major regulators of cell behavior, affecting polarized migration, cell division, vesicle trafficking and stem cell maintenance.

In summary, growing MT ends act as nanoplatforms for production of protein-protein interactions among the different +TIPs (Galjart, 2010).

3.5. Microtubule interactions with cortical sites

In 1986, Kirschner and Mitchison proposed a "search and capture" model for the attachment of MTs to cortical sites (Kirschner and Mitchison, 1986). This model proposes that dynamic MTs can be captured at specific sites, such as the membrane or the kinetochores, and be transiently stabilized. Nowadays, numerous examples of MT capture have been described and usually involve a +TIP protein that interacts with a specific cellular structure (Gundersen et al., 2004). These interactions are important to regulate different cellular processes. Examples include the capture of MTs at cortical sites to orientate the spindle during budding yeast division and the stabilization of MTs at the lamella of migrating fibroblasts, for polarized delivery of vesicles/factors. In both cases, the upstream regulator is a RhoGTPase, which through a Rho effector mediates the interaction between a +TIP and a cortical protein, leading to MT stabilization (Gundersen, 2002). MTs are also captured at FAs and AJs. Although, the mechanisms are not completely understood, there is a cooperative feedback relationship between MTs and cell adhesions: MTs affect the integrity of cell adhesions, whereas cell adhesion affects the dynamic properties of MTs (Akhmanova et al., 2009).

3.5.1. Microtubule interactions with Focal Adhesions

It has long been accepted that MTs are required for directional migration and that they are transiently associated with FAs at the leading edge of migrating cells, controlling the transport and recycling of integrins (Akhmanova et al., 2009). But, how is this connection established at the molecular level?

Three candidate +TIP proteins contribute to MT capture at FAs: APC, ACF7 and CLASPs (Stehbens and Wittmann, 2012) (Fig. 3). APC has been observed at clusters that localize close to FAs and contain the Wnt/β-catenin signaling molecule Dishevelled (Matsumoto et al., 2010). However, the relationship between Wnt signaling and APC function as a +TIP protein remains unexplored. ACF7 regulates FA dynamics by guiding MTs along actin filaments towards FA sites to regulate their disassembly (Ezratty et al., 2005) and this function may require the ATPase activity of ACF7 (Kodama et al., 2003; Wu et al., 2008). Its plus-end accumulation is regulated by GSK3β (Wu et al., 2011) and its cortical localization may be determined by interactions with APC (Zaoui et al., 2010). CLASPs seem to function downstream of ACF7 at FAs (Drabek et al., 2006). They attach MT plus-ends near

FAs via interaction with the PIP3-binding protein LL5β leading to MT stabilization (Hotta et al., 2010; Lansbergen et al., 2006). Thus, alterations in CLASPs lead to polarized migration defects (Drabek et al., 2006). It is clear that the three proteins are required for proper MT-FA interactions during cell migration. However, how they function together remains to be further explored.

3.5.2. Microtubule interactions with Adherens Junctions

As mentioned in previous sections, cadherins are well recognized for their ability to crosstalk with the actin cytoskeleton. Less attention has been paid at the dynamic interactions between AJs and MTs (Stehbens et al., 2009).

In 1995, MTs were shown to have an impact on AJs integrity. Treatment with MT depolymerizing drugs such as colchicine led to junctional discontinuities in a monolayer of thyroid cells (Yap et al., 1995). Years later, it was shown that not only MTs have an impact on AJs, but also AJs alter the dynamic properties of MTs. MTs that reached AJs in newt lung epithelial cells were more stable when compared with MTs at cell-free edges (Waterman-Storer et al., 2000). This was the first evidence of a feedback relationship between MTs and AJs. The presence of a capping protein at cell-cell contacts responsible for the increased MT stability was hypothesized, and the search for proteins implicated in MT capture at AJs began. The candidate MT binding proteins are ACF7, CLIP170, dynein and the minus-end binding protein Nezha (Fig.3):

MT growth towards sites of cell-cell adhesion could be guided by actin filaments. ACF7 seems to be involved in this process, since it binds both actin and MTs and it has been observed at sites of cell-cell adhesion in primary mKer (Karakesisoglou et al., 2000). Studies done in MCF-7 cells (human breast adenocarcinoma cell line) showed that cell-cell contact formation triggered the recruitment of CLIP-170 decorated MTs towards cadherin-based adhesions (Stehbens et al., 2006). However, the question remained as how MT plus-ends interact with AJs. Using PtK2 cells (rat kangaroo kidney epithelial cells) a colocalization between dynein and β-catenin was observed at cell-cell contacts, raising the possibility that the interaction between these two proteins tethers MTs to AJs (Ligon and Holzbaur, 2007; Ligon et al., 2001).

All proteins described so far are plus-end MT binding proteins and both minus-ends and plus-ends have been observed at cell-cell adhesions (Bellett et al., 2009). Indeed, cadherins stabilize MT minus-ends in cells lacking centrosomes (Chausovsky et al., 2000). A molecular link between AJs and MT minus-ends remained elusive until a seminal paper was published by the group of M. Takeichi showing MT minus-end anchorage to AJs via the interaction of p120 and the novel minus-end binding protein Nezha through an intermediate protein termed PLEKHA7 (Meng et al., 2008).

What could be the function of MTs at AJs? Two hypotheses have been proposed: transport of AJs proteins and formation of a scaffold for the concentration of cortical proteins (Akhmanova et al., 2009). Regarding the first hypothesis, the traffic of NCad to the membrane occurs in a kinesin-dependent manner (Mary et al., 2002). p120 seems to play a role in this process by interacting with

kinesins and MTs (Franz and Ridley, 2004; Yanagisawa et al., 2004). Supporting the second hypothesis, treatment of MCF-7 cells with low concentrations of nocodazole (which inhibit plus-end dynamics without depolymerizing MTs) impairs concentration of ECad molecules at the membrane, without altering their traffic to the surface (Stehbens et al., 2006).

How are these processes coordinated at the tissue level and how do they impact on the architecture of the tissue? The next section will focus on a specific epithelial tissue, the epidermis, with a particular focus on the MT - cell-cell adhesion crosstalk.

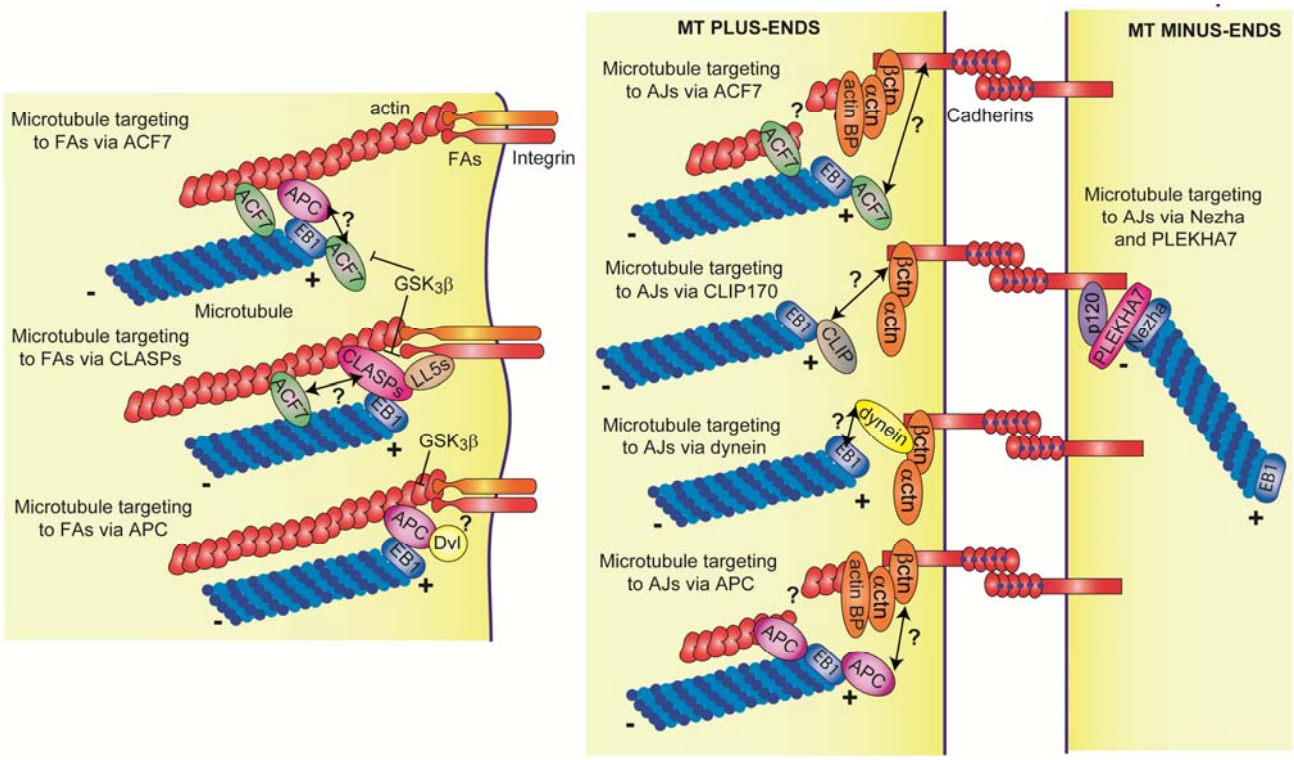


Figure 3 Proposed mechanisms of microtubule targeting to Focal Adhesions and Adherens Junctions. MTs move along actin filaments towards FAs via ACF7. CLASPs function downstream of ACF7 linking MTs to cortical proteins such as LL5s in the vicinity of FAs. APC has also been observed near FAs forming clusters that contain the Wnt pathway protein Dishevelled (Dvl). At AJs ACF7 may also function as a linker between actin and MTs. CLIP170 and APC have been observed at cell-cell contacts, but their cortical interacting partners remain unknown. Dynein interacts with βctn and this may represent a mechanism of MT plus-end targeting to AJs. MT Minus-ends are anchored to AJs via the interaction of p120 with PLEKHA7 and the MT minus-end binding protein Nezha.

4. The skin

The skin is the largest organ of our body and plays a fundamental role as a barrier protecting animals from external insults and dehydration (Fuchs and Horsley, 2008). It is composed of the epidermis (an ectoderm-derived epithelial tissue), the underlying dermis (a mesoderm derivative made of mesenchymal cells and ECM), the subcutaneous fat and sensory neurons. Epidermis and dermis are separated by a specialized ECM called the basement membrane, which represents a "niche" for progenitor epidermal cells (Ray and Lechler, 2011). The different cells of the skin

(keratinocytes of the epidermis, fibroblasts and immune cells of the dermis, melanocytes and adipocytes of the subcutaneous fat) maintain a continuous cross-talk fundamental for the maintenance of skin homeostasis. In addition, epidermis contains hair follicles and sebaceous glands (Fuchs, 2007).

4.1. Epidermal homeostasis

The epidermis is a self-renewing stratified epithelium composed of different layers of epithelial cells. Progenitor basal cells give rise to the differentiated layers of the epidermis, which commit to terminal differentiation and die and detach when they reach the surface of the tissue (Fuchs and Raghavan, 2002). This process of differentiation can be followed by the expression of specific markers (mainly specific types of keratins) in the different layers of the tissue. Thus, the epidermis is at the same time a robust structure that creates a mechanical and a chemical barrier, and a highly dynamic tissue, which undergoes continuous regeneration (Simpson et al., 2011).

The epidermis develops from a single layer of epithelial progenitor cells. At initial stages of development, these cells undergo symmetric cell divisions that expand the epithelial surface and generate more proliferative cells. Around embryonic day 13.5 in the mouse, basal mKer reorient the spindles, and divide asymmetrically, giving rise to differentiated stratified layers (Lechler and Fuchs, 2005; Poulson and Lechler, 2010). During subsequent stages of development, the balance between symmetric and asymmetric cell division is tightly regulated to fine-tune proliferation and differentiation. The crucial event that determines which kind of division will take place is the orientation of the spindle (Ray and Lechler, 2011). The underlying mechanism regulating this process involves polarity proteins, MT-associated proteins, cell signaling via the Notch pathway and cell-cell adhesion complexes (Lechler and Fuchs, 2005; Williams et al., 2011).

4.1.1. Cell-cell adhesion in the epidermis

The epidermis is not a classical polarized epithelium, with cells showing apical and basolateral membrane domains. Instead, it presents tissue-level polarization in terms of protein expression and architectural organization, which reflects the diverse functions of the different layers. Regarding adhesion complexes, hemidesmosomes and FAs are restricted to basal cells and attach the epidermis to the basement membrane. On the contrary, AJs, GJs and desmosomes are expressed in the different layers of the epidermis, although desmosomes are enriched in the spinous suprabasal layers. TJs are mainly restricted to the uppermost differentiated layers, and together with a specialized network of lipids and proteins, provide the barrier function to the tissue (Simpson et al., 2011).

The functional relevance of AJs proteins for the maintenance of epidermal homeostasis has been deciphered with the aid of conditional KO mouse models. Absence of both ECad and PCad leads to a loss of epidermal integrity, alterations in polarity proteins and loss of other junctional components, reflecting the key role of cadherins in the establishment of TJs, GJs and desmosomes (Sumigray et

al., 2012; Tinkle et al., 2004; Tinkle et al., 2008; Tunggal et al., 2005). β-catenin is fundamental for the formation of hair follicles (Huelsken et al., 2001) and α-catenin is indispensable to maintain AJs mediated adhesion. In addition, loss of α-catenin leads to hyperproliferation (Kobielak and Fuchs, 2006; Vasioukhin et al., 2001) and spindle orientation defects (Lechler and Fuchs, 2005). Regarding p120, it does not seem to play a major role in the maintenance of junctional integrity *in vivo* (most likely due to compensation by other members of the family). However, p120 deficiency in the epidermis overactivates RhoA which, via NFκB, generates an inflammatory response that progresses to squamous cell carcinoma (SCC) (Perez-Moreno et al., 2006). Moreover, RhoA overactivation impairs cytokinesis generating binucleated cells, which exhibit decreased proliferation *in vitro* (Perez-Moreno et al., 2008). How inflammation may impact on these genetically unstable cells *in vivo* remains to be elucidated.

Thus, cadherins and their associated catenins are critical for the maintenance of proper epidermal homeostasis (Perez-Moreno and Fuchs, 2006).

4.1.2. Microtubule network in the epidermis

As previously mentioned, cell differentiation is linked to MT reorganization. In simple epithelial cells, the formation of cell-cell contacts triggers the polarization of the MT network, from a radial centrosomal organization to a non-centrosomal polarized array of MTs (Musch, 2004). These MTs are organized vertically with their minus-ends apically, anchored at AJs (Meng et al., 2008), and their plus-end directed towards the basement membrane; although radial MTs that extend towards junctions have also been reported (Bellett et al., 2009; Stehbens et al., 2006). The mechanism leading to the formation of a non-centrosomal MT array in epithelial cells may be polymerization from the apical centrosome and subsequent release (Bartolini and Gundersen, 2006; Bellett et al., 2009). This MT organization is fundamental to maintain the polarized organization of epithelial cells. But, how is this polarized organization translated into a stratified epithelium such as the epidermis? In basal progenitor cells of the epidermis, the apical centrosome acts as a MTOC, from which MTs emanate towards cell-cell contacts. Upon differentiation, the centrosome is still able to nucleate MTs yet it does not function as a MTOC. Instead, anchoring proteins such as ninein or NDEL1, relocalize from the centrosome towards desmosomes in a desmoplakin-dependent manner to anchor MTs at areas of cell-cell contact (Lechler and Fuchs, 2007; Sumigray et al., 2011). These non-centrosomal MTs recruit type II myosins, strengthening AJs and promoting the formation of TJs and the epidermal barrier (Sumigray et al., 2012). Therefore, desmosomes play a major role in the reorganization of MTs during epidermal differentiation.

However, unsolved question is how MTs in basal progenitor cells of the epidermis interact with cell-cell adhesion sites, and how this interaction impacts on AJs stability (Fig. 4). Previous unpublished results from our laboratory identified a potential interaction between p120 and CLASP2 in a yeast two-hybrid screen. This link was especially interesting given the fact that p120 regulates both AJs

and MTs, whereas CLASP2 plays a role in MT stabilization specifically at cortical sites. This thesis project aims at validating the p120-CLASP2 interaction in keratinocytes and deciphering its functional relevance in the regulation of AJs.

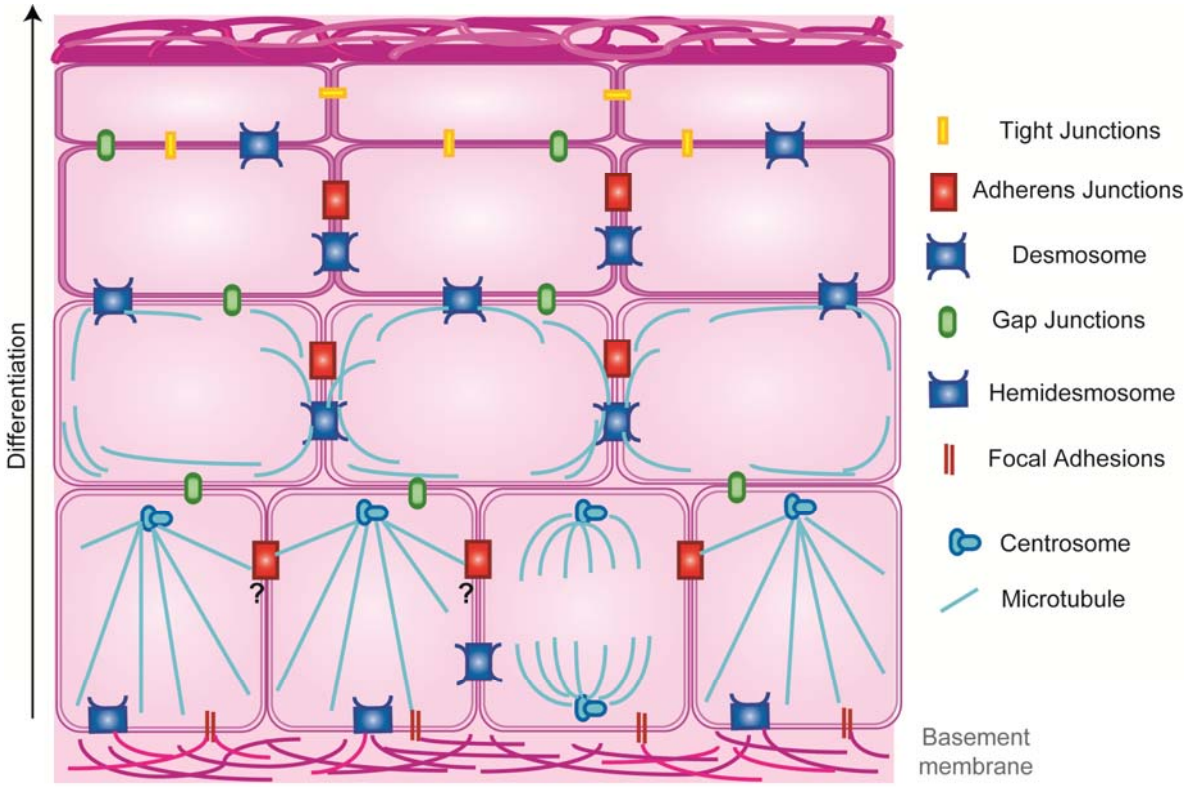


Figure 4 Connections between Microtubules and Adherens Junctions in the epidermis. In basal epidermal cells, the apical centrosome functions as the MTOC, generating a radial array of MTs that project towards sites of cell-cell adhesion. Upon differentiation, a non-centrosomal organization of MTs is generated, with MTs anchored at desmosomes. It remains to be addressed how microtubules in basal epidermal cells interact with cell-cell contacts.

Objectives

Classical cadherins and their connections with the MT cytoskeleton are important determinants of tissue organization and function. However, the molecular mechanisms for such an interaction are poorly characterized. Specifically, how MTs reach AJs in basal progenitor epidermal cells and what is the functional relevance of this link remains to be addressed. We hypothesize that the CLASP2-p120 interactions plays a role in MT targeting to AJs and preservation of cell-cell contacts. Moreover, this interaction could potentially impact on the polarized divisions of progenitor cells by controlling the orientation of the mitotic spindle. The specific aims of this thesis project are:

1. Validate and characterize the p120-CLASP2 interaction at AJs.
2. Determine the role of CLASP2 at AJs.
3. Investigate the consequences of the loss of CLASP2 in the proliferation/differentiation balance of keratinocytes.
4. Study the role of CLASP2 in the maintenance of epidermal homeostasis.

Objetivos

Las proteínas cadherinas y sus conexiones con el citoesqueleto de microtúbulos son factores determinantes para la organization y correcta funcionalidad de un tejido. Sin embargo, los mecanismos moleculares que median esta interación no están bien caracterizados. Específicamente, se desconoce cómo los microtúbulos interaccionan con las Uniones Adherentes en las células basales progenitoras de la epidermis, y cuál es la relevancia funcional de esta interacción. La hipótesis principal de este trabajo es que la interacción entre las proteínas CLASP2 y p120 desempeña un papel importante en la asociación de los microtúbulos con las Uniones Adherentes y en el mantenimiento de la adhesión celular. Además, esta interacción podría potencialmente estar implicada en las divisiones polarizadas de las células progenitoras, controlando la orientación del huso mitótico. Los objetivos de esta tesis son:

1. Validar y caracterizar la interacción entre las proteínas CLASP2 y p120 en las Uniones Adherentes.

2. Determinar qué papel desempeña CLASP2 en las Uniones Adherentes.

3. Investigar qué consecuencias tiene la pérdida de CLASP2 para el balance de proliferación y diferenciación en queratinocitos.

4. Estudiar el papel de CLASP2 en el mantenimiento de la homeostasis de la epidermis.

Materials and methods

1. Cell culture, transfection and viral infections

- 293T cells and L cells (ATCC CRL-2648) were cultured in DMEM (Gibco, Auckland) containing 10% FBS and antibiotics. 293-T cells were transfected with Lipofectamine 2000 (Invitrogen, California). 48 h after transfection the expression of the encoded proteins was analyzed by immunoblot.

- Primary mouse keratinocytes (mKer) were isolated from newborn mice backskins using dispase (Sigma, St. Louis) and trypsin (Gibco, Auckland). After filtration in 40 µm cell strainers, cells were cultured in Low Calcium Medium (0.05 mM Ca^{++}) -LC- containing 15% Ca^{++}-depleted FBS, as previously described (Nowak and Fuchs, 2009). mKer were transfected with Effectene reagent (Qiagen,Hilden) or X-tremeGENE DNA transfection reagent (Roche, Mannheim), according to the manufacturer's instructions.

Alternatively, mKer from adult mice were isolated from the tail. Briefly, tails were incubated in 0.75% trypsin (Gibco, Auckland) to allow the separation of the epidermis from the dermis, following treatment of the epidermis with 250 µg/mL of DNase (Sigma, St. Louis). Cells were filtered using 40 µm cell strainers and cultured in LC medium as described above.

To ablate p120 expression, p120 f/f mKer were infected O/N with 10^7 cfu of adenoviruses expressing Cre-GFP or GFP proteins (Cell Biolabs, San Diego) for two consecutive rounds of infection, to achieve *in vitro* recombination. Four days post-infection, cells were FACS-isolated according to their GFP expression levels, and cultured or processed for analyses.

CLASP2 expression was downregulated by infecting mKer O/N with lentiviruses expressing a CLASP2 specific shRNA (Clone TRCN0000183632, Sigma, St. Louis) in the presence of 6 µg/mL polybrene (Sigma, St. Louis). Lentiviruses were produced by cotransfecting 293T cells with the pLKO lentiviral plasmid and the packaging vectors psPax2 and pMD2.G. Two days after infection, cells were selected with 400 µg/mL of G418 (Calbiochem, Darmstadt). ECad KO-PCad shRNA mKer were kindly provided by E.Fuchs (Rockefeller University, NY, US) and have been described elsewhere (Tinkle et al., 2008).

- Human primary keratinocytes were obtained from the ATCC (PCS-200-010) and cultured in the absence of serum in CnT-57 medium (CellNTech, Bern) supplemented with L-glutamine.

- Human Squamous Carcinoma Cell lines (kindly provided by E. Wagner, CNIO, Madrid, Spain) and the mouse carcinoma cell line CarC were cultured in DMEM containing 10% FBS with antibiotics. Mouse carcinoma cell lines HaCa4 and PDV were cultured in DMEM/Ham12 containing 10% FBS,

and MCA3D cells in Ham12 containing 10% FBS with antibiotics. Epithelial mouse carcinoma cells were kindly provided by A. Cano, IIB, Madrid, Spain.

- Mouse embryonic fibroblasts (MEFs) were isolated from embryos at embryonic day 13.5 (E13.5) and cultured in DMEM containing 15% FBS and antibiotics. To ablate p120 expression in f/f MEFs, cells were infected with retroviruses encoding Cre-GFP (GFP was used as a control) in the presence of 6 μg/mL polybrene. Retroviruses were produced by cotransfecting 293T cells with the pBabe retroviral vector and the packaging pCL-ECO vector. Two-days after infection, MEFs were selected with 2 μg/mL of puromycin during 5 days, following FACS-Sorting for GFP to obtain a homogenous population of GFP-expressing cells.

-Mouse embryonic stem cells (ES cells) were used for the generation of the CLASP2 conditional KO mouse and cultured in gelatin-coated plates in DMEM containing 15% FBS and Leukemia Inhibitory Factor (LIF). CLASP2 expression was ablated *in vitro* in mouse f/+ ES cells using lentiviruses encoding Cre-GFP in the presence of 6 μg/mL polybrene. Lentiviruses were produced by cotransfecting 293T cells with the lentiviral plasmid pHFUW and the packaging vectors pMLDLg/pRRE, CMV-VSVG and RSV-Rev. Four days post-infection, cells were FACS-sorted according to their GFP expression levels and DNA was extracted from GFP^{high} and GFP^{low} ES cell populations.

2. Plasmids

For immunoflourescence and live imaging the following plasmids were used: GFP-CLASP2-α (gift of A. Akhmanova, Utrecht University, Utrecht, The Netherlands); EB3-GFP (gift of G. Gundersen, Columbia University, NY, US); p120-cherry (generated by cloning the cherry cDNA in frame at the C-term end of p120, in a Ker14 promoter vector) and Ker14-ECad-HA (Jamora et al., 2003) (gift of E. Fuchs, Rockefeller University, NY, US).

p120 deletion mutants were generated from the p120-cherry cDNA and cloned into the pHFUW lentiviral expressing vector (gift of. O Fernandez-Capetillo, CNIO, Spain) with a HA tag using the following forward primers:

p120FL FW: GAATTCATGGACGACTCAGAGGTGGA

p120ΔN FW: GAATTCATGCCCCCACCTTCAAAC

And the common reverse primer:

RV: GAATTC TTAAGCGTAGTCTGGGACGTCGTATGGGTAAATCTTCTGCATCAAGGGTGC.

For pull-down assays, equivalent deletion mutants were generated by PCR and cloned into pGEX-6P1 (GE-Healthcare, Uppsala).

CLASP2 deletion mutants were generated by PCR from the GFP-CLASP2-α cDNA and cloned into the pGEX-4T1 (GE-Healthcare, Uppsala) using the following primers:

CLASP2FL FW: TCCCCCGGGAATGGAGCCCCGCAGCATGGA

RV: TCCGCGGCCGCCTAACTTTGTCCAGAAACATCAGT

CLASP2ΔN FW: TCCCCCGGGAGGAAATCCTGCCAACAGTGC

RV: TCCGCGGCCGCCTAACTTTGTCCAGAAACATCAGT

CLASP2ΔC FW: TCCCCCGGGAATGGAGCCCCGCAGCATGGA

RV: TCCGCGGCCGCCTAAGAGCGTGGAGAGGAGTG

Southern Blot probes were generated by PCR using the Herculase polymerase (Stratagene, Santa Clara) and cDNA from pure B6 ES cells as a template. The following primers were used to amplify the probes:

5' probe FW: GGTGCATGGACAGACCCAGAGC; RV: CCTAGATAAAGGTGATAATGGG

3' probe FW: CTTCATTCCAGGCGAGTTTGCC; RV: CCACAGACCAACAGAAGAGGG

The PCR products were cloned into TOPO vector (Invitrogen, Camarillo).

3. Antibodies

The C-terminus of human CLASP2 (nucleotide positions 3074-3976 of the KIAA0627 cDNA) was cloned into pGEX4T1 as previously described (Akhmanova et al., 2001) and the resulting fusion protein, GST-hCLASP2, was expressed in BL21 competent bacteria (Novagen, Darmstad) and purified with Glutathione-Sepharose beads (GE Healthcare, Uppsala). This fusion protein was used to generate a CLASP2 rabbit polyclonal antibody (Covance, New Jersey), which was affinity-purified using the GST-hCLASP2 fusion protein crosslinked to Glutathione-Sepharose columns.

The following antibodies were also used: acetylated-tubulin mouse mAb (T7451, Clone 6-11B-1, Sigma, St. Louis), α-catenin rabbit pAb (C2081, Sigma, St. Louis), α-tubulin mouse mAb (T9026, Clone DM1A, Sigma, St. Louis), , β-actin mouse mAb (A5441, Clone AC-15, Sigma, St. Louis), CLASP1 rabbit pAb (13713-1-AP, Proteintech, Chicago), EB1 mouse mAb (610534, Clone 5/EB1, BD Biosciences, New Jersey), ECad rat mAb (13-1900, Clone ECCD2, Invitrogen, Camarillo), Filaggrin

rabbit pAb (PRB-417P, Covance, New Jersey), γ-tubulin mouse mAb (T6557, Clone GTU-88, Sigma, St. Louis), GFP rabbit pAb (A11122, Invitrogen, Paisley), Glu-tubulin rabbit pAb (AB3201, Chemicon International, Temecula), GST mouse mAb (88D/G4), HA rat mAb (11867431001, Clone 3F10, Roche, Mannheim), HA mouse mAb (2367, Clone 6E2, Cell Signaling Technology, Danvers), HA rat mAb (1867423, Clone 3F10, Roche, Mannheim), Integrin β4 chain (CD104) rat mAb (553745, Clone 346-11A, BD Biosciences, San Jose), Keratin 1 rabbit pAb (PRB-165P, Covance, New Jersey), Keratin 10 rabbit pAb (PRB-159P, Covance, New Jersey), NCad rat mAb (13-2900, Clone NCD-2, Invitrogen, Paisley), Nezha rabbit pAb (gift of M. Takeichi, RIKEN Center for Developmental Biology, Japan), p120-catenin CT mouse mAb (33-9600, Clone 15D2, Invitrogen, Camarillo), p120-catenin NT mouse mAb (33-9700, Clone 6H11, Invitrogen, Camarillo), pericentrin rabbit pAb (PRB-432C, Covance, New Jersey) and tyrosinated α-tubulin rat mAb (MCA77G, Clone YL1/2, AbD Serotec, Oxford).

4. Flow cytometry and cell sorting

- Integrin-α6 sorting: mKer were isolated from the backskin of newborn mice as mentioned above. Cell suspensions were stained for 30 min at 4ºC using a phycoerythrin-conjugated rat anti-CD49f (integrin-α6 chain) mAb (555736, Clone GoH3, BD Pharmigen, San Jose) as previously described (Nowak and Fuchs, 2009). Cells were sorted on a FACSAria Ilu using the CellQuest Pro software (BD Biosciences, San Jose).

- GFP sorting: mKer infected with adenoviruses expressing GFP were trypsinized and cell suspensions were sorted on a FACSAria Ilu as described above.

- Cell cycle analysis: cells were resuspended in staining solution containing 0.1% sodium citrate dehydrate, 0.1% triton, 200 mg/mL RNAse A and 10 mg/mL propidium iodide (Sigma, St. Louis), and analyzed using a FACSCanto and the FlowJo software (TreeStar).

5. RNA isolation and Real Time-PCR (RT-PCR)

Total RNA was isolated from cells using TRIZOL (Invitrogen, California). 2 μg were used for cDNA synthesis using the Ready to Go You Prime It First-Strand beads and random primers (GE Healthcare, Uppsala). RT-PCR reactions were conducted using the GoTaq qPCR Master Mix (Promega, Wisconsin) and a MasterCycler Ep-Realplex thermal cycler (Eppendorf, Hamburg). The following settings were used: 2 min at 95° C for initial denaturing, 35 cycles of 15 s at 95°C denaturing, 40 s at 57°C annealing, and 45 s at 72°C extension. The expression levels of PCR products were normalized according to GAPDH. The following primers were used:

Primer pair	Forward	Reverse
Total CLASP2	TTGTCGTCCTCTGTCAGTGC	TGCCACGTCTTCTGTCTGTC
CLASP2-α	AAAGAGACATTCCCCCTGCT	TTGCACTGCTGACAGGATTC
Keratin 5	CAGTGTGCCAACCTCCAGAACG	AGCCCGCTACCCAAACCAAGAC
Loricrin	TCACTCATCTTCCCTGGTGCTT	GTCTTTCCACAACCCACAGGA
GAPDH	CGTAGACAAAATGGTGAAGGTCGG	AAGCAGTTGGTGGTGCAGGATG

6. Proliferation and apoptosis assays

A double thymidine block was performed to synchronize mKer at the G1/S transition. After each block with 2mM thymidine (Sigma, St. Louis) cells were washed with a release solution containing 10 µM deoxycytidin (Sigma, St. Louis). Cells were fixed to analyze spindles 5 hours after the second thymidine block, since at this time point the highest number of mitotic figures was found.

To analyze cell proliferation equal amounts of mKer were plated in triplicates and cells were counted every day using a Neubauer chamber. To analyze apoptosis, TUNEL positive cells were detected by immunofluorescence using the In Situ Cell Death Detection Kit (Roche, Mannheim) according to the manufacturer's instructions.

For colony formation assays 300, 500 or 800 cells were plated in fibronectin (Merck, New Jersey) coated plates. One week after plating, cells were fixed with 4% PFA (Electron Microscopy Science, Hatfield) and stained with crystal violet.

Cell size was evaluated by staining mKer with cell mask (Invitrogen, California).

7. Immunofluorescence, immunohistochemistry and cell treatments

Embryos and organs were frozen and embedded in OCT compound. Sections (8 µm) were fixed in -20ºC methanol for 2 minutes for immunofluorescence stainings.

For immunohistochemistry, embryos and organs were fixed with formaldehyde and embedded in paraffin. Sections (5 µm) were deparaffinized following standard protocols and stained for CLASP2.

Cells were plated on coverslips and fixed in -20 ºC methanol for 2 minutes. Fixed cells were blocked using a blocking buffer containing: 0.3% triton, 1% BSA, 5% Normal Goat Serum (NGS), 5% Normal Donkey Serum (NDS) and 1% gelatin in PBS. Cells were incubated with primary antibodies according

to the manufacturer's instructions, followed by incubation with fluorescence conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Pennsylvania).

For extraction of monomeric tubulin, cells were washed with a MT stabilizing buffer -MSB- (85 mM PIPES, 1 mM EDTA, 1 mM $MgCl_2$, 2 M glycerol) and extracted with 200 µg/mL of saponin dissolved in MSB for 5 minutes, prior to fixation in -20ºC methanol.

To fully depolymerize MTs, cells were treated for 4 h with 30 µM nocodazole (Sigma, St. Louis). To determine the population of nocodazole resistant MTs, cells were treated for 30 min with 5 µM nocodazole, followed by extraction of monomeric tubulin as previously described.

Images were acquired in a Leica TCS-SP5 (AOBS) confocal microscope using oil immersion objective 63X HCX PL APO 1.4 N.A. with LAS AF v 2.5 software.

8. Cell adhesion assays

mKer were switched from LC Medium to a medium containing 2 mM Ca^{++} (Normal Calcium Media -NC-) to evaluate AJs formation at different time points. AJs disassembly was evaluated by washing cells with PBS, followed by incubation in LC medium.

Plates were coated with 0.1 µg/µL of ECad-FC recombinant protein (Sigma, St. Louis) as previously described (Chappuis-Flament et al., 2001). Both Scramble controls and CLASP2-deficient mKer were trypsinized with 0.02% crystalline trypsin (Biological Industries, Beit-Haemek) and allowed to attach to the ECad-coated plates for 30 min and 1 h, followed by cold methanol fixation.

9. Live imaging microscopy

To monitor the dynamic movement of CLASP2 to the cortex, WT mKer were transfected with constructs encoding GFP-CLASP2 and p120-cherry. Cells were switched to NC medium for 5 h. Images were taken at intervals of 2 s in a TCS-SP5 (AOBS) laser scanning confocal microscope with a water immersion objective 63X HCX PL APO 1.2 N.A.

To monitor EB3 dynamics at cell-cell contacts, mKer were transfected with a plasmid encoding EB3-GFP and switch to NC medium for 5 h. Images were acquired every second in a Nikon Eclipse Ti fluorescent microscope with a oil immersion objective 63X Apo TIRF 60x NA1.49, 0.13-0.21 DIC**.** Analysis of the EB3 trajectories was done using Imaris software (Bitplane Scientific Software, Zurich).

For Fluorescence Recovery After Photobleaching (FRAP) experiments mKer were transfected with the p120-cherry encoding construct and switched to Normal Calcium Media for 5 h. A constant ROI

was defined at the membrane and bleached with a 561 nm laser at 100%. Time-lapse images were acquired every 3 s for a total of 64 s. To calculate FRAP curves, a constant region demarcating the membrane was defined within the initial ROI. Bleaching was controlled by examining the fluorescence intensity over time of an unbleached region. Images were taken in a TCS-SP5 (AOBS) confocal microscope with a water immersion objective 63X HCX PL APO 1.2 N.A using a temperature-controlled incubator chamber in the presence of CO_2. The data was normalized to pre-bleach and post-bleach values and fitted to the one phase exponential equation

$$Fluorescence\ recovery = \frac{F(t) - F(0)}{F(-t) - F(0)} = Mf \cdot (1 - e^{-\tau t}) \qquad -\tau = \frac{Ln0.5}{t1/2}$$

F(t) is the average fluorescence intensity of the selected membrane region within the ROI, F(0) is the post-bleach fluorescence intensity, F(-t) is the pre-bleach fluorescence intensity, Mf is the mobile fraction and t1/2 is the half life.

In every case 10 µM HEPES was added to the medium to maintain a constant pH.

10. Protein purification and *in vitro* pull-down assays

Recombinant GST-p120 and GST-CLASP2 proteins were expressed in Arctic RIL competent bacteria (Stratagene, Santa Clara) to avoid degradation of the recombinant proteins and purified with Glutathione-Sepharose beads. Subsequently, GST-p120 fusion proteins were treated O/N with 2 units of PreScission Protease (GEHealthcare, Uppsala) per 100 µg of recombinant protein to remove the GST domain. Pull-downs were carried out with 1 µg of each recombinant protein in a buffer containing: 50 mM Tris pH 7, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% Triton X-100 and PMSF, and samples were analyzed by immunoblot. Alternatively, GST-p120 fusion proteins were used to pulled-down a lysate of 293-T cells transfected with different CLASP2 deletion mutants (Mimori-Kiyosue et al., 2005). Equal amounts of GFP-tagged proteins were used for the pull-downs.

Surface membrane proteins were isolated from mKer using a Cell Surface Protein Isolation Kit (Thermo Scientific, Waltham) following the manufacturer's instructions.

11. Immunoprecipitations and immunoblotting

Immunoprecipitations were done as previously described (Meng et al., 2008). Briefly, mKer lysates were prepared with a buffer containing 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1.5 mM $MgCl_2$, 1mM DTT and 0.5% Triton X-100. Lysates were incubated O/N with the corresponding antibodies followed by 1 h incubation with protein G- or A-Sepharose. Proteins were detected by immunoblot following standard procedures.

For analysis of total protein levels by immunoblotting cells were lysed in RIPA buffer containing: 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.2% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and 0.2% SDS.

12. Centrosomal isolation

The procedure to isolate centrosomes has been previously described (Meigs and Kaplan, 2008). Briefly, cells were treated with 10 µg/mL nocodazole and 5 µg/mL cytochalasin B (Sigma, St. Louis) and lysed in a hypotonic buffer containing 1 mM Tris-HCl pH 8 and 0.5% Triton X-100. Centrosomes were isolated using Ficoll 400 (Sigma, St. Louis) following purification with a sucrose gradient (20%-62.5%). 0.5 mL fractions were collected from the gradient and analyzed by SDS-PAGE. Centrosomal fractions were identified based on the presence of γ-tubulin.

13. Southern Blotting

For analysis of homologous recombination in ES cell clones, 20 µg of DNA were digested using the corresponding restriction enzymes O/N. The digested DNAs were run on a 0.7% agarose gel following depurination of the gel with HCl and denaturation with 0.5 M NaOH and 1.5 M NaCl. The DNA was transferred O/N to a nylon membrane (Macherey-Nagel, Düren) by capillarity in SSC 10x.

Probes were labeled with the Prime-It Random Primer Labeling Kit (Stratagene, Santa Clara) in the presence of radioactive ^{32}P αdCTP following the manufacturer's instructions. Radioactive probes were incubated with the membranes O/N using the following hybridization buffer: 23.6 mM Na_2HPO_4, 18 mM NaH_2PO_4, 900 mM NaCl, 2.5 mM EDTA pH 8, 320 mM polyvinylpirrolidone, 0.8 mg/mL Ficoll 400, 10 mg/mL SDS, 50 mg/mL dextran sulphate and 12.5 M formamide. The autoradiography was done using a Phosphor screen (GE Healthcare, Uppsala).

14. Generation of CLASP2 conditional KO mouse

A targeting vector was designed to flank with LoxP sites the first common exon to all three CLASP2 isoforms as well as the first exon of isoforms β and γ (exons 8 and 9). The targeting vector was generated by Gene Bridges (Heidelberg).

After linearization with XhoI, the targeting vector was electroporated into B6/129 ES cell clones. Clones were screened for homologous recombination by Southern Blot as described later in the text. Positive clones were aggregated into CD1 embryos and germ-line transmission was verified by Southern Blot and genotyping. The following primers were used for the genotyping:

LoxP5: FW: GCATACTCCGGAAGGAGCCTCC; RV: TGCCCTAGGTGCTGCAACAGCC

LoxP3: FW: TGCAGCATAGAAAGTGTCTGCC; RV: GGTTTACTTGATCACCATCAGCC

A founder flox/+ (f/+) mouse was used to generate the CLASP2 f/f line. The deletion of the gene was achieved by crossing the animals to K5-Cre expressing mice (kindly provided by E. Wagner, CNIO, Spain) and analyzed by PCR with the following primers:

Δ band pair 1: FW: CTTTCTGATGCAGTAGCACCC; RV: ACAGTCCTGAAATACCACTCCC

Δ band pair 2: FW: TGTGGTAAAGCTGCTTCTCTGCC; RV: GGCATCTGAAACGAAAGTACCC

15. Quantification and statistical analysis

The levels of ECad, p120 and other AJs components at the membrane were analyzed using ImageJ software. Briefly, individual plot profiles were generated randomly and the maximum level of fluorescence intensity was quantified.

The levels of CLASP2 at the membrane, colocalizing with ECad, were analyzed with imageJ. Random individual plot prolifes were generated at AJs. The maximum CLASP2 fluorescence intensity corresponding to the maximum peak of the ECad fluorescence intensity profile was quantified. For MT quantifications individual MTs were manually counted in cell-cell contact areas as well as cell free edges and represented as number of MTs per μm. The EB3-GFP time-lapse microscopy experiments were analyzed using the Imaris software (Bitplane Scientific Software, Zurich). Individual EB3-GFP comets were identified using the automatic tools of the Imaris software, and individual tracks were manually quantified.

In every set of experiments all immunofluorescence images were taken under the same exposure conditions. For statistical analysis, the normality of the data was evaluated with a Kolmogorov-Smirnov test. Data that did not present a Gaussian distribution was analyzed using the Mann-Whitney U test. Data that presented a Gaussian distribution but unqueal variances was analyzed using a Student's t test with a Welch's correction.

Results

1. Validate and characterize the p120-CLASP2 interaction at Adherens Junctions

1.1. CLASP2-p120 interaction at Adherens Junctions in mouse keratinocytes

1.1.1. Generation of a CLASP2 rabbit polyclonal antibody

To characterize the p120-CLASP2 interaction we first needed to generate a CLASP2 rabbit polyclonal antibody, since at the time this project was started there were no commercial CLASP2 antibodies available. The antibody was raised against the conserved C-terminal region as previously described (Akhmanova et al., 2001). The fusion protein GST-hCLASP2 was expressed in DL21 bacteria and purified with glutathione sepharose beads (Fig. 5a). This fusion protein was used to immunize rabbits and the resulting serum was purified by affinity. First, the antibody concentration was titrated by Western Blot showing a single band at a dilution of 1/500 with a lysate of mouse keratinocytes (mKer) (Fig. 5b). The specificity of the antibody was verified by peptide-competition assays, in which the antibody was incubated in the presence of either 5 µg of GST (as a control) or 5 µg of GST-hCLASP2. By Western Blot, the band corresponding to CLASP2 was not detected in a lysate of mKer when the antibody was co-incubated with GST-hCLASP2 (Fig. 5c). The signal observed by immunofluorescence of mouse backskin sections was also specific since it was lost when the antibody was co-incubated with GST-hCLASP2 (Fig. 5d). Knock-down experiments of CLASP2 in mKer also confirmed the specificity of the antibody as will be described later on in the text.

As mentioned before, CLASP1 and CLASP2 are homologous proteins with a high degree of similarity. In particular, the C-terminal sequence of CLASP2 used to generate the antibody has a 73% degree of homology with CLASP1. Since CLASP1 and CLASP2 have also very similar molecular weight, it was important to evaluate whether our CLASP2 polyclonal antibody cross-reacted with CLASP1. To this end we over-expressed GFP-CLASP1 in 293T cells and evaluated by Western Blot whether the signal obtained with a GFP Western Blot colocalized with a CLASP2 Western Blot. As a control, GFP-CLASP2 was also overexpressed. We could observe that the CLASP2 polyclonal antibody was able to recognize the overexpressed GFP-CLASP1, although to a lesser extent than GFP-CLASP2. Thus, there is a weak cross-reactivity with CLASP1. This has also been observed with a published homemade CLASP2 polyclonal antibody (Akhmanova et al., 2001).

In conclusion, we were able to generate a CLASP2 polyclonal antibody as a useful tool to explore and characterize the interaction between p120 and CLASP2.

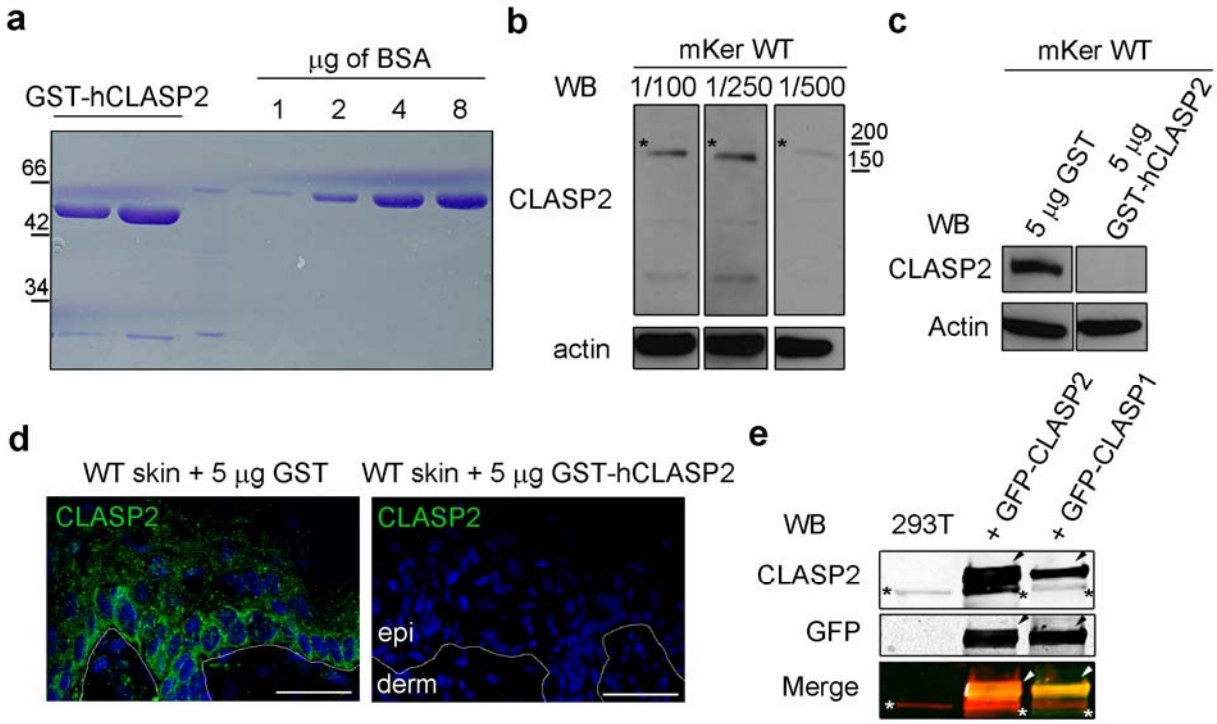


Figure 5 Generation of a CLASP2 rabbit polyclonal antibody. (a) Purified recombinant GST-hCLASP2 was run on a SDS-PAGE and subsequently stained with Coomassie staining solution. A BSA standard was used to quantify the amount of GST-hCLASP2 purified. **(b)** Tritiation of the CLASP2 antibody purified by affinity. A lysate of mKer was analyzed by Western Blot using different concentrations of the CLASP2 antibody. The asterisk marks the band corresponding to the expected molecular weight of CLASP2 (170KDa). **(c)** Peptide competition assay: the CLASP2 antibody was used in the presence of either 5 µg of GST or 5 µg of recombinant GST-hCLASP2 to analyze by Western Blot a lysate of mKer. **(d)** Skin sections from newborn WT mice were stained with the CLASP2 antibody in the presence of either 5 µg of GST or 5 µg of recombinant GST-hCLASP2. epi: epidermis, derm: dermis. Scale bar, 25 µm. **(e)** Western Blot of 293T cells transfected with either GFP-CLASP2 or GFP-CLASP1. The bands corresponding to the CLASP2 WB are marked in red and those corresponding to the GFP WB in green. Asterisks indicate endogenous CLASP2. Arrowheads indicate transfected GFP-CLASP.

1.1.2. CLASP2 and p120 interact at Adherens Junctions

The results obtained in the yeast-two hybrid screening pointed towards CLASP2 as a potential candidate to mediate microtubule (MT) interactions with AJs via p120. To validate this hypothesis, we analyzed CLASP2 distribution in primary mKer treated with calcium to induce the formation of cell-cell contacts. Under these conditions CLASP2 was enriched at AJs, where it colocalized with p120 (Fig. 6a). This colocalization was evident when p120 and CLASP2 fluorescence intensities at AJs were represented in a plot profile (Fig. 6b). We also observed an enrichment of CLASP2 in the perinuclear region, which most likely corresponds to the Golgi apparatus as previously described (Efimov et al., 2007).

To analyze whether this CLASP2 enrichment was specific for areas of cell-cell contact we used two different approaches. First, we cultured primary mKer at low density, in such a way that small colonies were formed. In absence of calcium in the culture media AJs were not formed, and CLASP2 localized to the ends of MTs (the characteristic localization pattern of a +TIP protein) (Fig. 6c). After treatment with calcium, CLASP2 was recruited to areas of cell-cell contact as previously mentioned. Some of the AJs-associated CLASP2 seemed to be also associated to MTs that projected towards ECad puncta. At cell-free edges CLASP2 retained a MT plus-end localization, without any obvious enrichment (Fig. 6c). We further analyzed CLASP2 distribution in ECad KO – PCad-deficient primary mKer. After calcium treatment, ECad-dependent cell-cell contacts were not formed, and CLASP2 did not present any cortical enrichment, but retained its MT plus-end localization (Fig. 6d). Thus, CLASP2 enrichment at sites of cell-cell adhesion depends on the formation of cadherin-based cell-cell contacts. However, these experiments do not definitely show whether AJs are directly required for CLASP2 localization at cell-cell contacts, since double ECad KO – Pcad-defiencient mKer also fail to form desmosomes and TJs *in vitro* (Tinkle et al., 2008). Therefore, to specifically test whether cadherin-based adhesion directly recruits CLASP2 to AJs, we coated plates with recombinant Fc-ECad (extracellular domain of murine ECad) and allowed WT mKer to attach for 1 hour. In this way, cells attach to the plates exclusively via ECad-mediated ligation. Mouse Fc fragments were used as a control for the specificity of the attachment, as well as cells plated in the presence of EDTA. Confocal imaging of CLASP2 and p120 in the XZ plane allowed us to observe an enrichment of both proteins in some regions of the basal side of the attached cells (Fig. 6e). This result indicates that cadherin-based cell-cell adhesion can directly recruit CLASP2 to AJs.

To analyze the dynamic behaviour of CLASP2 at sites of cell-cell adhesion in WT mKer we used time-lapse confocal videomicroscopy. We were able to observe GFP-CLASP2 comets moving continuously towards regions of cell-cell adhesion (as marked by p120-cherry), in a movement typical of MT +TIP proteins. Upon reaching these areas, many of these CLASP2 comets travelled along the AJs, suggesting an interaction of CLASP2 with junctional components (Video 1).

In conclusion, so far we have seen that cadherin-based adhesion leads to the recruitment of CLASP2. Upon reaching cell-cell contacts, CLASP2 interacts with AJs components, including p120.

To validate the CLASP2-p120 interaction biochemically, CLASP2 was immunoprecipitated from mKer treated with calcium. CLASP2 immunoprecipitates (IP) contained p120 and ECad (Fig. 7a). Thus, CLASP2, p120 and ECad form a ternary complex in mKer. However, with this experimental approach

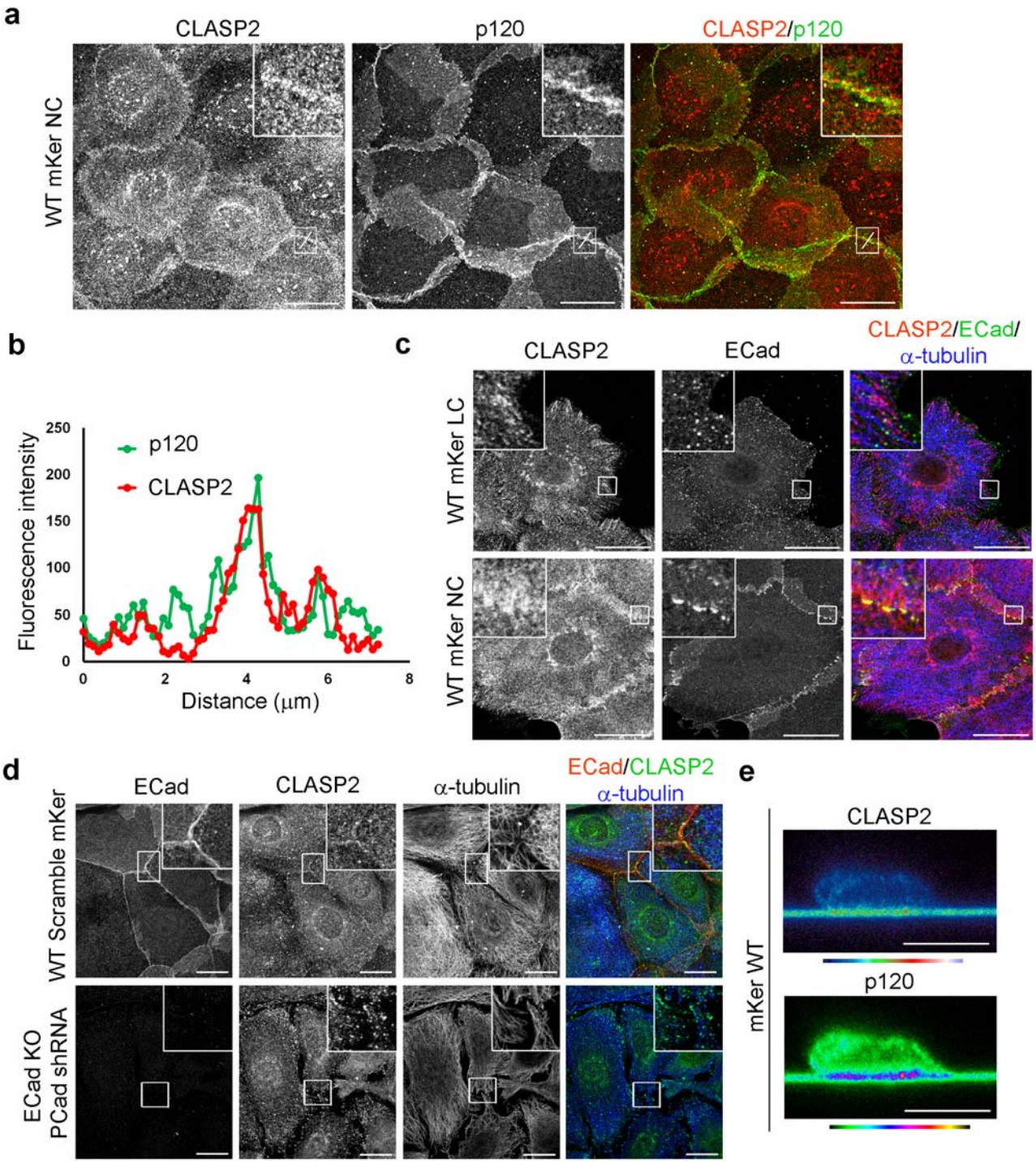


Figure 6 CLASP2 and p120 interact at AJs. (a) WT primary mKer immunostained for CLASP2 and p120 in the presence of calcium (NC: normal calcium) to induce formation of AJs. Scale bar, 25 μm. **(b)** Plot profile of CLASP2 and p120 fluorescence intensity at AJs in the area indicated in panel a. **(c)** WT primary mKer were grown in low confluency to allow the formation of colonies. Cells were immunostained for CLASP2, ECad and α-tubulin, in the presence (NC) or absence of calcium (LC: low calcium). Scale bar, 25 μm. **(d)** WT and ECad KO - PCad shRNA mKer immunostained for ECad, CLASP2 and α-tubulin. Scale bar, 25 μm. **(e)** WT primary mKer were plated in Fc-ECad-coated plates and allowed to attach for 1 hour. The attached cells were immunostained for CLASP2 and p120, and XZY pictures were taken. Scale bar, 10 μm.

we could not conclude whether the interaction takes place at cell-cell junctions, or alternatively, in cytoplasmic vesicles during the process of vesicular traffic. To address this question biochemically, we purified biotin-labeled membrane proteins using a streptavidin-based purification. This procedure does not disrupt interactions between transmembrane and cytoplasmic proteins, and thus allowed us to recover whole ECad complexes. From the lysate of purified surface proteins, we immunoprecipitated CLASP2 and we were able to detect in the immunoprecipitates not only CLASP2, but also p120 and ECad (Fig. 7b). A lysate of mKer non-treated with biotin was used as a control of the purification.

In conclusion, CLASP2, p120 and ECad form a complex at AJs in mKer *in vitro* as demonstrated biochemically and by immunofluorescence.

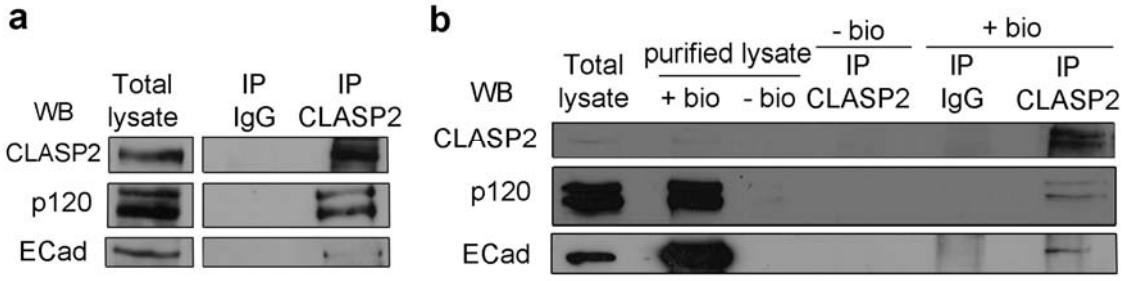


Figure 7 CLASP2, p120 and ECad form a complex at AJs in mKer. (a) CLASP2 was immunoprecipitated from mKer treated with calcium and the immunoprecipitates (IP) were analyzed for CLASP2, ECad and p120 by immunoblot. Rabbit IgGs were used as a control. **(b)** Cell surface proteins from mKer treated with calcium were labelled with biotin and purified with a streptavidin column. CLASP2 was immunoprecipitated from the purified lysate of surface proteins, and CLASP2, p120 and ECad were analyzed by immunoblot. Rabbit IgGs were used as a control of the immunoprecipitation, and a lysate of cells without biotin was used as a control of the purification. bio: biotin.

1.1.3. The interaction between CLASP2 and p120 at Adherens Junctions is maintained after disruption of microtubules

We have seen that CLASP2 is targeted to AJs upon contact formation, where it interacts with p120. But, once CLASP2 and p120 interact at the membrane, are MTs required to sustain this interaction at AJs? To answer this question, we used the MT depolymerizing drug nocodazole.

As a first step, we needed to control that we were able to fully depolymerize the MT network. To this end, we first treated mKer with 30 µM nocodazole for different time points and then extracted the monomeric tubulin using a non-ionic detergent. This approach allows the specific detection of polymerized tubulin, since monomeric tubulin is lost from the cell (Gundersen et al., 1987). In this way, we were able to determine that a treatment of 4 hours with 30 µM nocodazole was sufficient to fully depolymerize all MTs in our system (Fig. 8a).

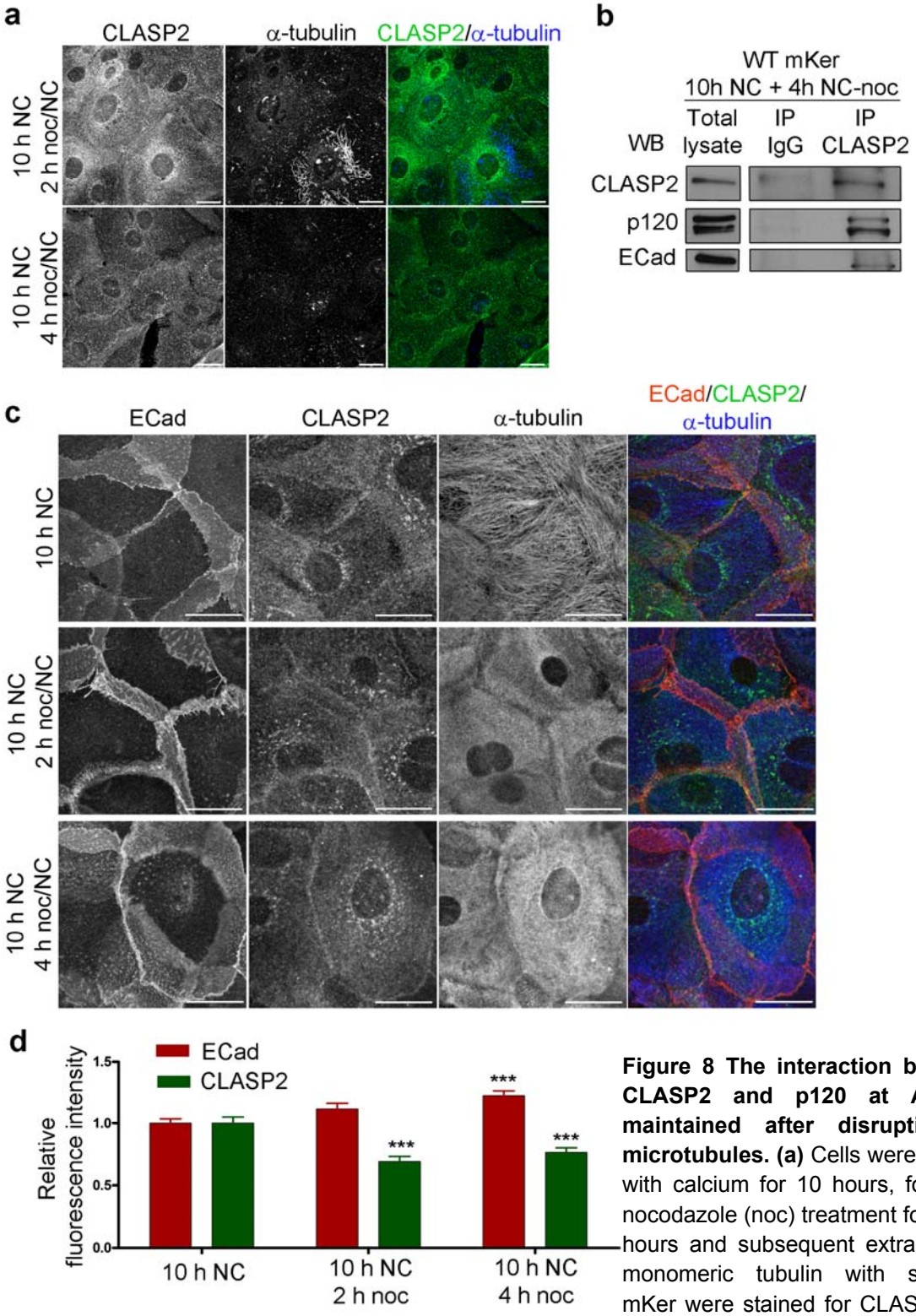


Figure 8 The interaction between CLASP2 and p120 at AJs is maintained after disruption of microtubules. (a) Cells were treated with calcium for 10 hours, following nocodazole (noc) treatment for 2 or 4 hours and subsequent extraction of monomeric tubulin with saponin. mKer were stained for CLASP2 and α-tubulin. Scale bar, 25 μm. **(b)** CLASP2 was immunoprecipitated from mKer treated for 10 hours with calcium following 4 hours treatment with nocodazole in the presence of calcium. CLASP2 immunoprecipitates were analyzed by Western Blot for CLASP2, ECad and p120. **(c)** mKer were immunostained for ECad, CLASP2 and α-tubulin at different time points of a nocodazole treatment. Scale bar, 25 μm. **(d)** Random individual plot profiles at AJs (n= 10 per cell/40-50 cells) were obtained for the different time points of nocodazole treatment. The maximum fluorescence intensity of ECad and CLASP2 for each profile was quantified and normalized to the control non-treated with nocodazole. Data is represented as mean ± s.e.m.; *** $p < 0.0001$, U Mann-Whitney for ECad; *** $p < 0.001$, Student's t test for CLASP2.

Equally to the experiments that validated the p120-CLASP2 interaction, we immunoprecipitated CLASP2 from mKer which, in this case, had been treated with 30 µM nocodazole for 4 hours after AJs formation. Strikingly, CLASP2 immunoprecipitates contained not only CLASP2, but also p120 and ECad (Fig. 8b). This experiment indicates that the p120-CLASP2 interaction can be maintained in absence of MTs. We next evaluated CLASP2 localization by immunofluorescence, and observed that, at all time points analyzed, CLASP2 remained at AJs despite the absence of MTs (Fig. 8c); although its levels dropped 30% compared to a control without nocodazole and measured in terms of fluorescence intensity at AJs (Fig. 8d). Concomitantly, ECad levels at the membrane were stabilized upon nocodazole treatment (Fig. 8c, d), in line with the previously reported requirement of MTs for ECad internalization (Ivanov et al., 2006). Thus, the CLASP2-p120 interaction does not require MTs.

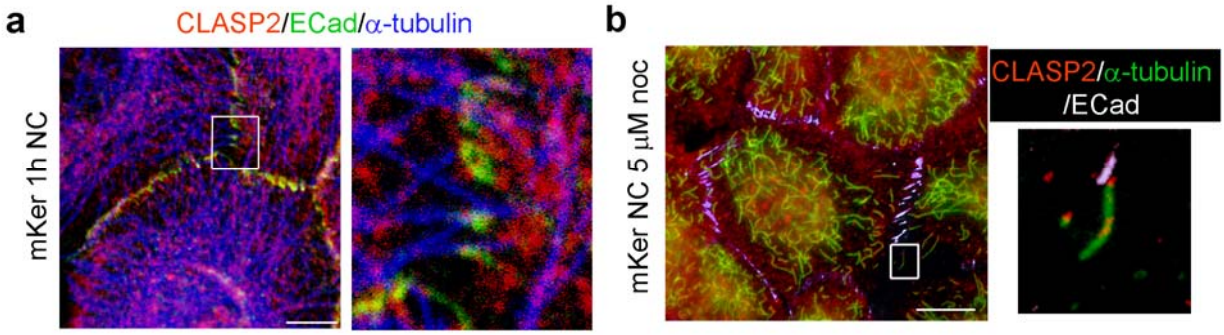


Figure 9 CLASP2-decorated MTs reach cadherin-based adhesions. (a) WT mKer were treated with calcium for 1 hour and immunostained for CLASP2, ECad and α-tubulin. The right image is an inset of the square region. Scale bar, 7.5 µm. **(b)** WT mKer were treated with calcium for 4 hours, following treatment with 5 µM nocodazole for 30 minutes and extraction of monomeric tubulin with saponin. Cells were immunostained for ECad, CLASP and α-tubulin. The right image is an inset of the square region. Scale bar, 25 µm

These experiments raised the question of whether CLASP2 was able to interact simultaneously with MTs and p120. Since the MT network at AJs is very dense, we treated WT mKer for 1 hour with calcium and focused our attention on AJs areas with a lower MT density. Following this approach, we observed CLASP2-decorated MTs reaching cadherin punta (Fig. 9a), suggesting that CLASP2 may bridge MTs with cadherin-based adhesions. To further distinguish individual MTs, we treated mKer with 5 µM nocodazole for 30 min, which is known to depolymerize non-stable MTs, exposing the resistant ones. Under these conditions, we were able to distinguish individual MTs, which, in most of the cases, were decorated by CLASP2 comets (Fig. 9b). In addition, a population of resistant MTs was found associated to AJs via their plus-ends decorated with CLASP2 (Fig. 9b). These observations

suggest that MTs are targeted to AJs via the interaction of CLASP2 with junctional components, most likely p120.

These experiments indicate that there may be two pools of CLASP2 at AJs: a population of CLASP2 molecules that mediates connections between AJ components and MTs, and a second population of CLASP2 that is able to interact with AJs components in a MT independent fashion. This could explain the two different dynamic behaviors of CLASP2 observed by time-lapse microscopy (Video1). As mentioned before, some CLASP2 molecules move towards AJs and disappear when they reach the cell-cell contacts, whereas other CLASP2 molecules move along the AJs, indicative of an interaction with AJs components.

1.1.4. CLASP2 can be recruited to cell-cell contacts in absence of microtubules

We have seen that CLASP2 can be maintained at AJs even after the MT network is depolymerized. We also wondered whether CLASP2 could relocalize to AJs during contact formation in the absence of MTs. To test this possibility we first depolymerized the MT network with a 4 hours treatment of 30 µM nocodazole, and then induced AJs formation adding calcium to the medium in the presence of nocodazole. Importantly, nocodazole treatment by itself (in the absence of calcium) did not lead to AJs formation in our system (Fig. 10a), in disagreement with previous observations made in human keratinocytes (Kee and Steinert, 2001). Under these conditions, CLASP2 did not show a clear cortical enrichment (Fig. 10a). We next immunoprecipitated CLASP2 from mKer treated with nocodazole for 4 hours and then switched to a medium with calcium for another 4 hours maintaining the nocodazole. Surprisingly, CLASP2 immunoprecipitates contained CLASP2, p120 and ECad (Fig. 10b).

Immunofluorescence analysis revealed that CLASP2 could be detected at AJs already 1 hour after addition of calcium in mKer that formed cell-cell contacts in the absence of MTs (Fig. 10c). The levels of CLASP2 at AJs after 1 hour in calcium were comparable to those found in mKer non-treated with nocodazole (Fig. 10d). At later time points of contact formation, whereas control mKer progressively increased their levels of CLASP2 at AJs, mKer treated with nocodazole showed significantly reduced levels of CLASP2 when compared to controls (Fig. 10c, d). These experiments suggest that CLASP2 can be recruited to AJs in absence of MTs, although this process is less efficient than in the presence of MTs. ECad-based cell-cell contacts initially formed to the same extent in control cells and nocodazole treated cells, indicating that MTs are not required for the initial steps of contact formation. Hower, at later time points, we detected a significant increase in the levels of ECad at the membrane in nocodazole treated cells (Fig. 10d), in line with our previous results

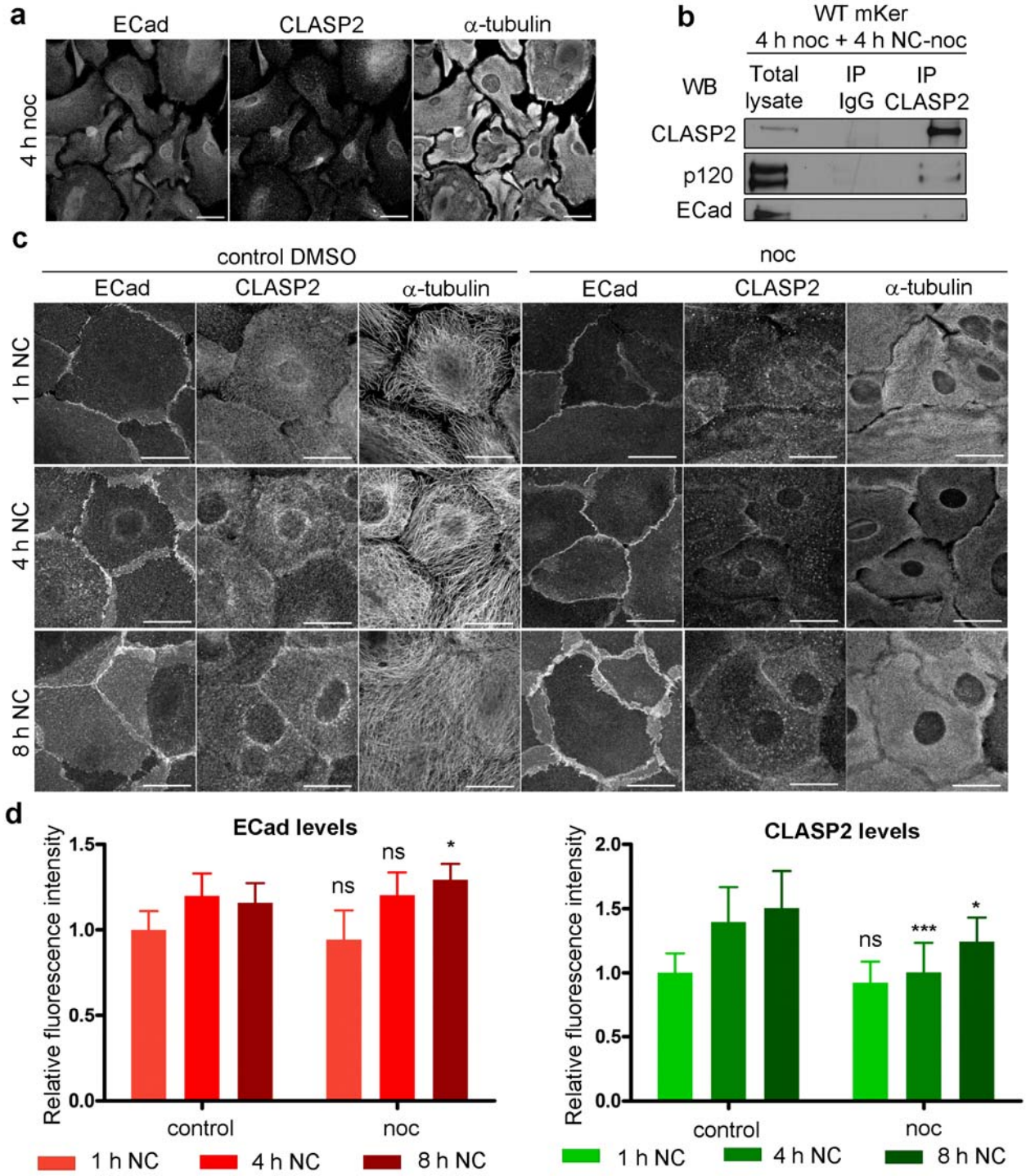


Figure 10 CLASP2 can relocalize to AJs in the absence of MTs (a) mKer were treated with 30 µM nocodazole (noc) for 4 hours in the absence of calcium. Cells were immunostained for ECad, CLASP2 and α-tubulin. Scale bar, 25 µm. **(b)** CLASP2 was immunoprecipitated from mKer treated with noc for 4 hours and switch to a normal calcium medium (NC) with noc for another 4 hours. The immunoprecipitates were immunoblotted for CLASP2, p120 and ECad. **(c)** mKer treated with noc for 4 hours were switched to a medium containing calcium for 1 hour, 4 hours or 8 hours. Cells were immunostained for ECad, CLASP2 and α-tubulin. Scale bar, 25 µm. **(d)** Random individual plot profiles (n= 10 per cell/40-50 cells) were generated to quantify ECad and CLASP2 fluorescence intensity at AJs. The maximum fluorescence intensity of ECad and the corresponding CLASP2 fluorescence intensity for each profile were quantified. Data is normalized to the control non-treated with nocodazole after 1 hour of calcium and represented as mean ± s.e.m.; * $p < 0.02$, *** $p < 0.001$, Student's t test (comparison done with the corresponding control non-treated with nocodazole).

showing a possible role of MTs during ECad internalization. Thus, in mKer lacking MTs, CLASP2 is recruited less efficiently to areas of cell-cell contact. AJs form normally but the maintenance of AJs dynamics may be affected by an impairment of ECad internalization.

1.1.5. p120 deficiency impairs CLASP2 localization to Adherens Junctions

Our results indicate that CLASP2 localizes to AJs and forms a complex with ECad and p120, which is maintained even in absence of MTs. But, is p120 responsible for recruiting CLASP2 to cell-cell contacts? To answer this question we took advantage of the p120 conditional KO mouse model available in the laboratory. In these mice the expression of Cre recombinase is under the control of the Keratin 5 promoter, and thus p120 is lost in epithelial tissues, such as the epidermis (Perez-Moreno et al., 2006). We isolated primary mKer from the backskin of newborn mice and after evaluating the genotyping of the pups, we validated by immunofluorescence that the expression of p120 was lost (Fig. 11a). We also confirmed that p120 deficiency leads to a delay in AJs formation in mKer *in vitro* as previously described (Perez-Moreno et al., 2006) (Fig. 11a). When we analyzed CLASP2 localization by immunofluorescence we observed that CLASP2 was lost from cell-cell contacts in absence of p120 (Fig. 11a). Quantification of CLASP2 fluorescence intensity at AJs showed a 50% decrease in the levels of CLASP2 at AJs in absence of p120 (Fig. 11b). On the other hand, CLASP2 properly localized to the plus-ends of MTs (Fig. 11c) suggesting that p120 deficiency only alters the pool of CLASP2 that localizes to cell-cell contacts.

These results were validated biochemically. To this end, flox/flox mKer were isolated from the backskin of newborn mice and infected *in vitro* with either adenoGFP as a control (p120 f/f) or adenoCre-GFP (p120 Δ/Δ) to ablate the expression of p120. Cells were further FACS-Sorted according to their GFP expression levels to obtain a homogenous population of infected cells. Western Blot analysis showed that the efficiency of the deletion was very high (Fig. 11d). After immunoprecipitation of ECad, we observed that CLASP2 was present in ECad complexes in control mKer, but not in p120-null mKer, indicating that p120 is required for CLASP2 to associate with ECad-based cell-cell adhesion complexes (Fig. 11e). Treatment of mKer with calcium for a longer period of time (12 hours) led to recruitment of CLASP2 to cell-cell contacts (Fig. 12a, b), suggesting that additional mechanisms may participate in the association of CLASP2 with cadherin-based adhesions.

As it was mentioned before, p120 deficiency leads to the internalization of cadherins from the membrane (Davis et al., 2003; Ireton et al., 2002). Therefore, it was plausible that CLASP2 did not

localize to AJs at early time points of contact formation in p120-null mKer due to the decreased levels of ECad in the membrane, and not as a direct consequence of p120 deficiency. To test this

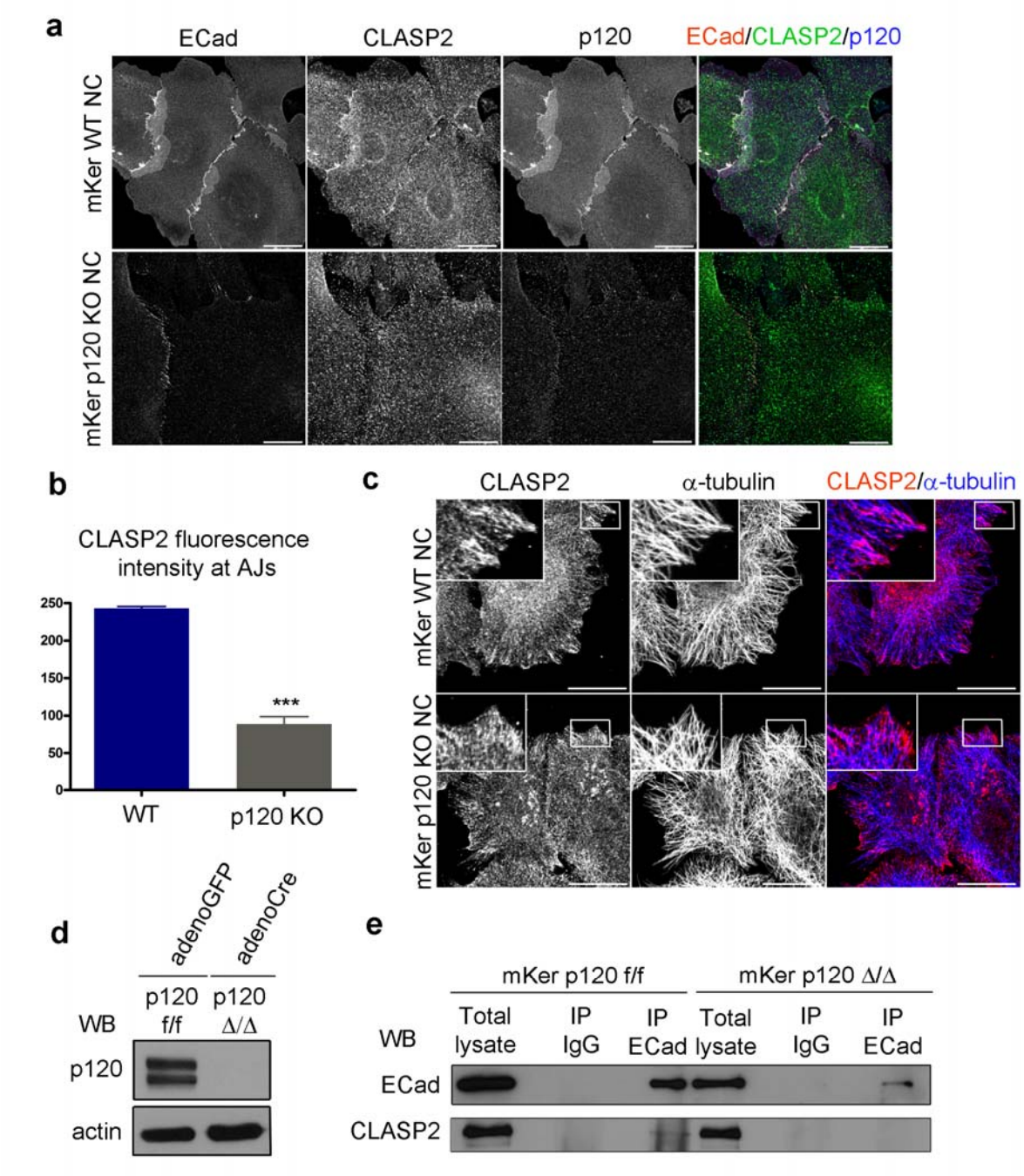


FIGURE 11 p120 is required for CLASP2 localization to AJs (a) WT and p120 KO primary mKer immunostained for ECad, CLASP2 and p120 in the presence of calcium (NC). Scale bar, 25 μm. **(b)** Random individual plot profiles (n = 10 per cell / 5 cells) were obtained from WT and p120 KO mKer. The fluorescence intensity level of CLASP2 corresponding to the maximum ECad level in each profile was quantified and normalized to the cytoplasmic CLASP2 fluorescence intensity. Data is normalized to WT levels and represented as mean ± s.e.m.; *** $p < 0.0001$, Mann Whitney U test. **(c)** WT and p120 KO primary mKer grown in low confluency and immunostauned for CLASP2 and α-tubulin in the presence of calcium (NC). Scale bar, 25 μm. **(d)** Western Blot showing the levels of p120 in primary flox/flox mKer infected either with adenoGFP (p120 f/f) as a control, or adenoCre-GFP (p120 Δ/Δ). **(e)** ECad was immunoprecipitated from control flox/flox adeno-GFP infected mKer (p120 f/f) or from p120-null mKer infected with adenoCre-GFP (p120 Δ/Δ). The immunoprecipitates were blotted for ECad and CLASP2.

possibility, we over-expressed ECad-HA to promote its accumulation at the membrane in both control and p120-null mKer. Of note, previous results have shown that ECad overexpression is able to rescue epithelial morphology even in absence of p120 (Ireton et al., 2002). Immunofluorescence analyses of CLASP2 showed that despite the presence of ECad at the membrane in both control and

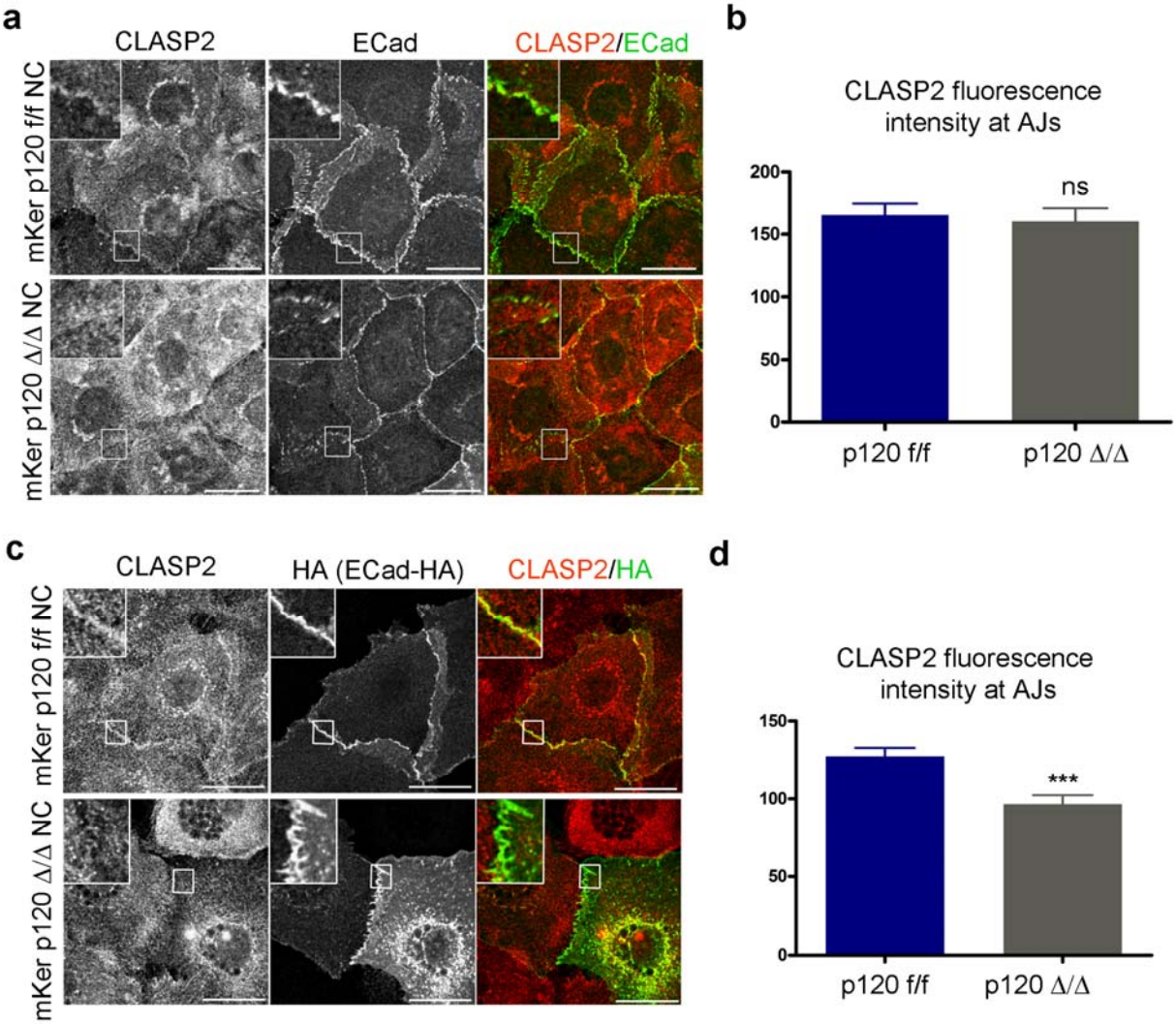


Figure 12 CLASP2 can be recruited to AJs via p120-independent mechanisms (a) Control (p120 f/f) mKer and p120-null (p120 Δ/Δ) mKer treated with calcium for 12 hours and immunostained for ECad and CLASP2. Scale bar, 25 μm. **(b)** Random individual plot profiles were generated in both p120 f/f and p120 Δ/Δ mKer. The point of maximum ECad fluorescence intensity was identified and the CLASP2 fluorescence intensity associated to this value was quantified and normalized to the cytoplasmic CLASP2 fluorescence intensity. Data is normalized to control values and represented as mean ± s.e.m. ns: non-significant. **(c)** ECad-HA was overexpressed in control (p120 f/f) and p120-null (p120 Δ/Δ) mKer. Cells were switched to calcium-containing media and immunostained for CLASP2 and HA. Scale bar, 25 μm. **(d)** Random individual plot profiles were generated in both p120 f/f and p120 Δ/Δ mKer. The point of maximum ECad-HA fluorescence intensity was identified and the CLASP2 fluorescence intensity associated to this value was quantified and normalized to the cytoplasmic CLASP2 fluorescence intensity. (n = 16 cells, 5 profiles per cell, 3 independent experiments). Data is normalized to control values and represented as mean ± s.e.m.; *** $p < 0.0003$, Student's t test.

p120-null mKer[1], CLASP2 did not significantly colocalize with ECad in absence of p120 when compared to controls (Fig. 12c). Certain areas at cell-cell contacts were able to concentrate CLASP2 in the absence of p120, but in most of the cases cadherin-based adhesions failed to efficiently recruit CLASP2 (Fig. 12c). These results were quantified, and although the levels of CLASP2 at AJs were higher in p120-null cells overexpressing ECadherin (Fig. 12d) than in p120-null mKer (Fig. 11b), there was still a significant difference between control ECad-overexpressing and p120-null ECad-overexpressing mKer (Fig. 12d).

Collectively these results indicate that p120 is responsible for CLASP2 localization to AJs; although other mechanisms may co-operate to ensure CLASP2 targeting to AJs. As a proof-of-principle of our hypothesis, it was important to re-express p120FL in our p120-null mKer to rescue the phenotype. Not only that, but also we wondered whether a mutant form of p120 unable to bind CLASP2 but able to rescue the ECad phenotype, could rescue CLASP2 localization to AJs. To answer this question, we needed to map the domains responsible for the p120-CLASP2 interaction, which led us to characterize biochemically the CLASP2-p120 interaction (objective 1.2.).

1.1.6. CLASP1 localizes to Adherens Junctions in a p120-independent manner

As it was mentioned in the introduction, CLASP2 and CLASP1 are homologous proteins that share many biological functions (Galjart, 2005). Thus, it was important to test whether CLASP1 was also able to bind p120 and localize to AJs. To analyze CLASP1 we used a commercially available rabbit polyclonal antibody, but we were unable to efficiently immunoprecipitate CLASP1 from mKer. For this reason we overexpressed GFP-tagged CLASP1 together with p120-HA in 293T cells (due to the low efficiency of transfection of mKer). p120-HA was weakly detected in GFP-CLASP1 immunoprecipitates (Fig. 13a), maybe via CLASP2, since CLASP1 is part of a +TIP protein complex with CLASP2 (Mimori-Kiyosue et al., 2005).

Next we looked at CLASP1 localization in mKer treated with calcium. We observed that CLASP1 localized to AJs, and this localization was maintained even in the absence of p120, in contrast to CLASP2 (Fig. 13b). Therefore, CLASP1 seems to localize to AJs via a p120-independent mechanism, and since we had no evidences of a possible direct interaction with p120, we focused our subsequent studies on CLASP2.

[1] Note the presence of many vesicles containing ECad in the cytoplasm of p120-null cells as a consequence of the decreased stability of cadherins at the membrane.

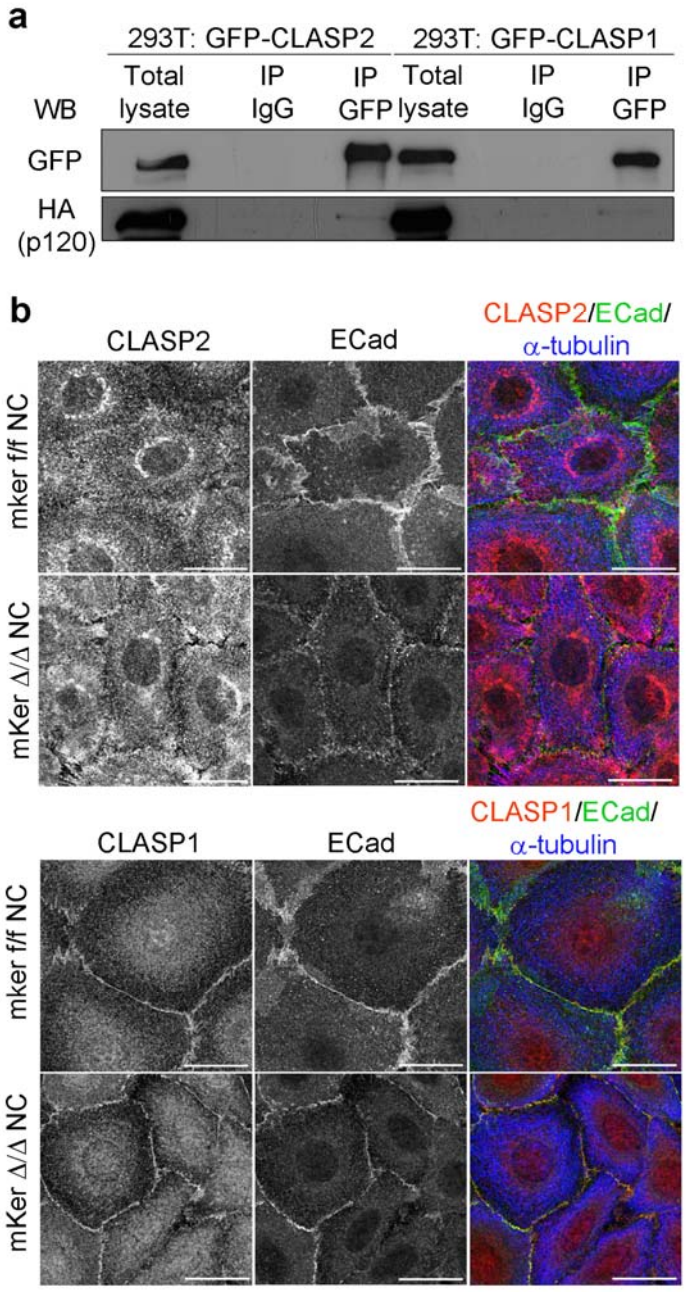


Figure 13 CLASP1 localizes to AJs in a p120-independent manner (a) 293T cells were transfected with p120-HA and either GFP-CLASP2 or GFP-CLASP1. GFP immunoprecipitates were blotted for GFP (as a control) and HA. **(b)** Control adenoGFP infected (p120 f/f) and p120-null adenoCre-GFP infected (p120 Δ/Δ) mKer treated with calcium were immunostained for ECad, α-tubulin and either CLASP2 or CLASP1. Scale bar, 25 μm.

1.2. Biochemical characterization of the CLASP2-p120 interaction

1.2.1. CLASP2 and p120 directly interact

We have seen that CLASP2 and p120 form a complex in mKer together with ECad. But, is their interaction direct? To answer this question we performed *in vitro* pull-down assays with purified recombinant proteins. First, we cloned full-length p120 and CLASP2 in pGEX vectors, which allow expression of recombinant GST-tagged proteins in bacteria. The full-length proteins were expressed in Arctic bacteria, and purified with glutathione sepharose beads (Fig. 14a). In the case of p120, it

was necessary to cleave apart GST, to avoid non-specific interactions with GST-CLASP2. To do so, GST-p120 was treated with the PreScission protease and purified p120 was recovered in the supernatant (Fig. 14b). This purified p120 was able to specifically interact with GST-CLASP2 in a pull-down assay (Fig. 14c). Thus, we can conclude that the interaction between CLASP2 and p120 is direct.

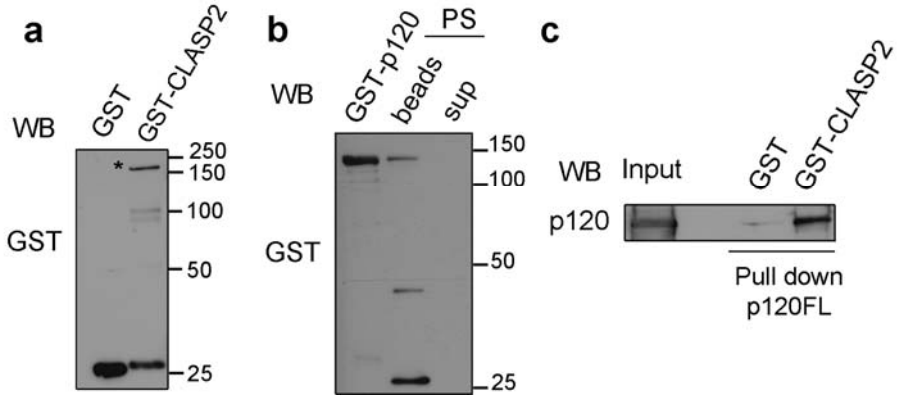


Figure 14 CLASP2 and p120 directly interact. (a) Purified GST and GST-CLASP2 (asterisk) were immunoblotted for GST. **(b)** GST immunoblot showing the digestion of GST-p120 with PreScission protease (PS). Cleaved p120 was recovered in the supernatant (sup) and GST remained bound to the beads. **(c)** Pull-down assay using p120FL as input and GST-CLASP2, following immunoblot for p120.

1.2.2. CLASP2 and p120 interact via the N-terminal domain of p120 and the N1 region of CLASP2

With the final aim of performing rescue experiments, we decided to map the domains responsible for the p120-CLASP2 interaction. The yeast-two hybrid screen indicated that the p120 N-terminal domain was involved in the interaction with CLASP2. For this reason, we first generated a mutant p120 protein lacking the N-terminal domain (p120ΔN) as well as the p120 N-terminal domain alone (p120N) (Fig. 15a). For CLASP2 we had no previous information regarding the possible domain involved in the interaction with p120, and we began our analyses by eliminating both the N-terminal domain (CLASP2ΔN) and the C-terminal domain (CLASP2ΔC) (Fig. 15a).

Purification of all recombinant GST-tagged CLASP2 and p120 constructs was done as previously described in the text (Fig. 15b). p120FL was able to interact with all the CLASP2 constructs generated, whereas p120ΔN was not pulled-down with CLASP2FL, validating the results of the yeast-two hybrid screen (Fig. 15c). Thus, the N-terminal domain is necessary to interact with

CLASP2, but is it sufficient? Pull-down experiments using p120N showed that indeed p120N was sufficient to interact with the three CLASP2 constructs generated (Fig. 16a). Finally, it was important

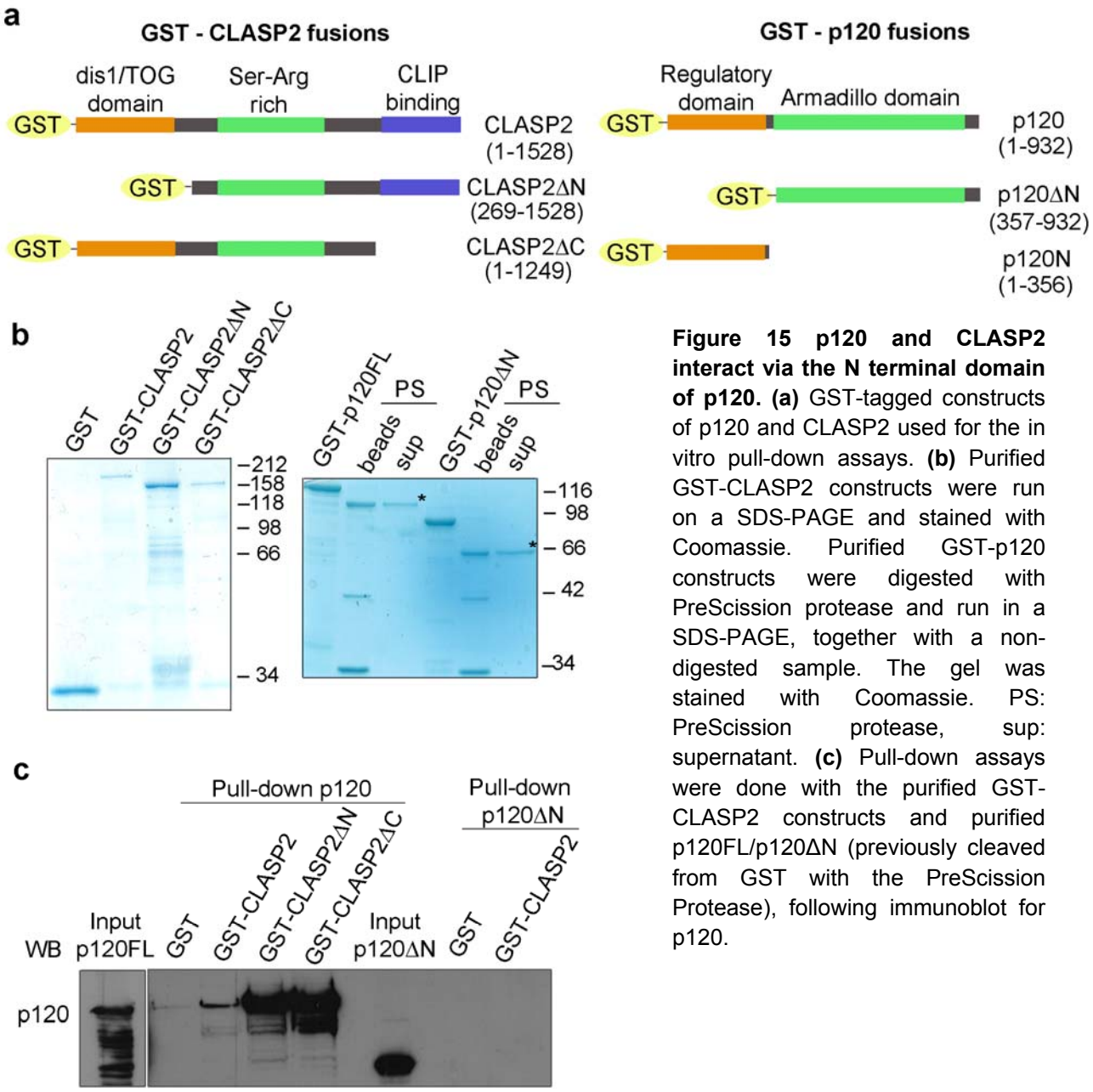


Figure 15 p120 and CLASP2 interact via the N terminal domain of p120. (a) GST-tagged constructs of p120 and CLASP2 used for the in vitro pull-down assays. **(b)** Purified GST-CLASP2 constructs were run on a SDS-PAGE and stained with Coomassie. Purified GST-p120 constructs were digested with PreScission protease and run in a SDS-PAGE, together with a non-digested sample. The gel was stained with Coomassie. PS: PreScission protease, sup: supernatant. **(c)** Pull-down assays were done with the purified GST-CLASP2 constructs and purified p120FL/p120ΔN (previously cleaved from GST with the PreScission Protease), following immunoblot for p120.

to demonstrate whether the endogenous p120 expressed in mKer was able to interact with recombinant CLASP2. For this reason we used a lysate of mKer treated with calcium as input of the pull-down, and once again we detected an interaction between endogenous p120 and all three CLASP2 constructs generated (Fig. 16b). We have demonstrated that the N-terminal domain of p120 is necessary and sufficient to interact with CLASP2, and on the other hand, CLASP2 interacts with p120 through its mid region. However, we could not resolve with more precision the specific

CLASP2 domain involved in the p120 interaction with our set of constructs. Thus, we used a second set of constructs kindly provided by Dr. Anna Akhmanova (Utrecht University, Utrecht, The Netherlands) (Fig. 17a). The GFP-tagged constructs were expressed in 293T cells and equivalent amounts of the recombinant proteins were used to do a pull-down with purified GST-p120N (GST was used as a control) (Fig. 17b, c). Following this approach we were able to observe that the GFP-CLASP2N1 construct strongly interacted with p120N. This construct contains the Ser-Arg rich region, responsible for binding to MTs and EB1, as well as a cryptic TOG-like domain (Slep, 2009).

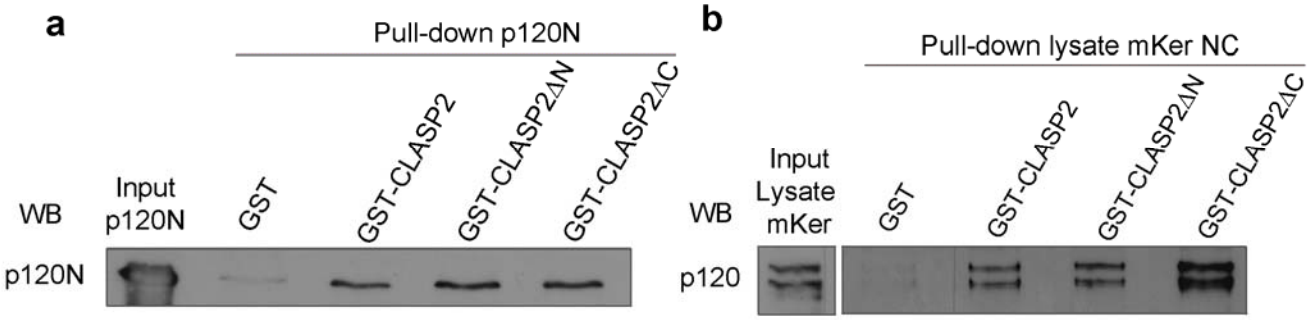


Figure 16 The N-terminal domain of p120 mediates the interaction with CLASP2. (a) Pull-down assay with the purified GST-CLASP2 constructs and purified p120N, following immunoblot for p120N. **(b)** Pull-down assay with the purified GST-CLASP2 constructs and a lysate of mKer previously treated with calcium, following immunoblot for p120.

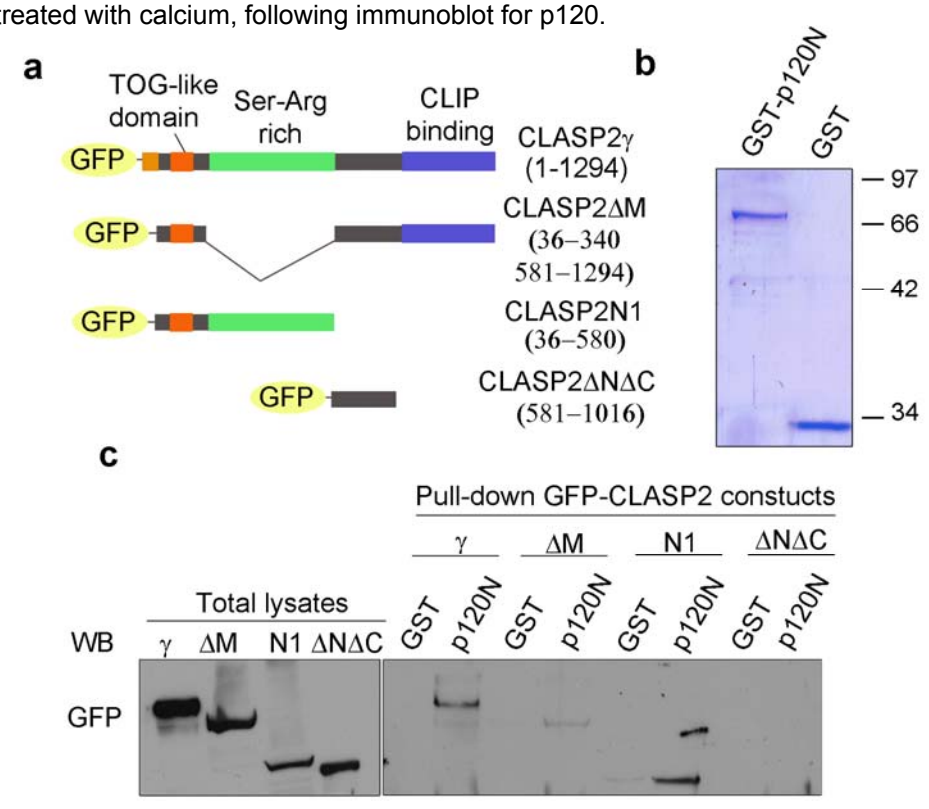


Figure 17 The CLASP2 N1 region interacts with p120 (a) Schematic representation of the GFP-tagged CLASP2 constructs used for the pull-down. **(b)** Coomassie staining of purified GST and GST-p120N. **(c)** Purified GST and GST-p120N were incubated with lysates of 293T cells expressing the GFP-tagged constructs indicated in each case. The pulled-down proteins were blotted for GFP.

The GFP-CLASP2ΔM, which lacks the Ser-Arg rich region, interacted weakly with p120N. This result indicates that the region between aa 36-340 (which contains a TOG-like domain) is sufficient to bind p120N.

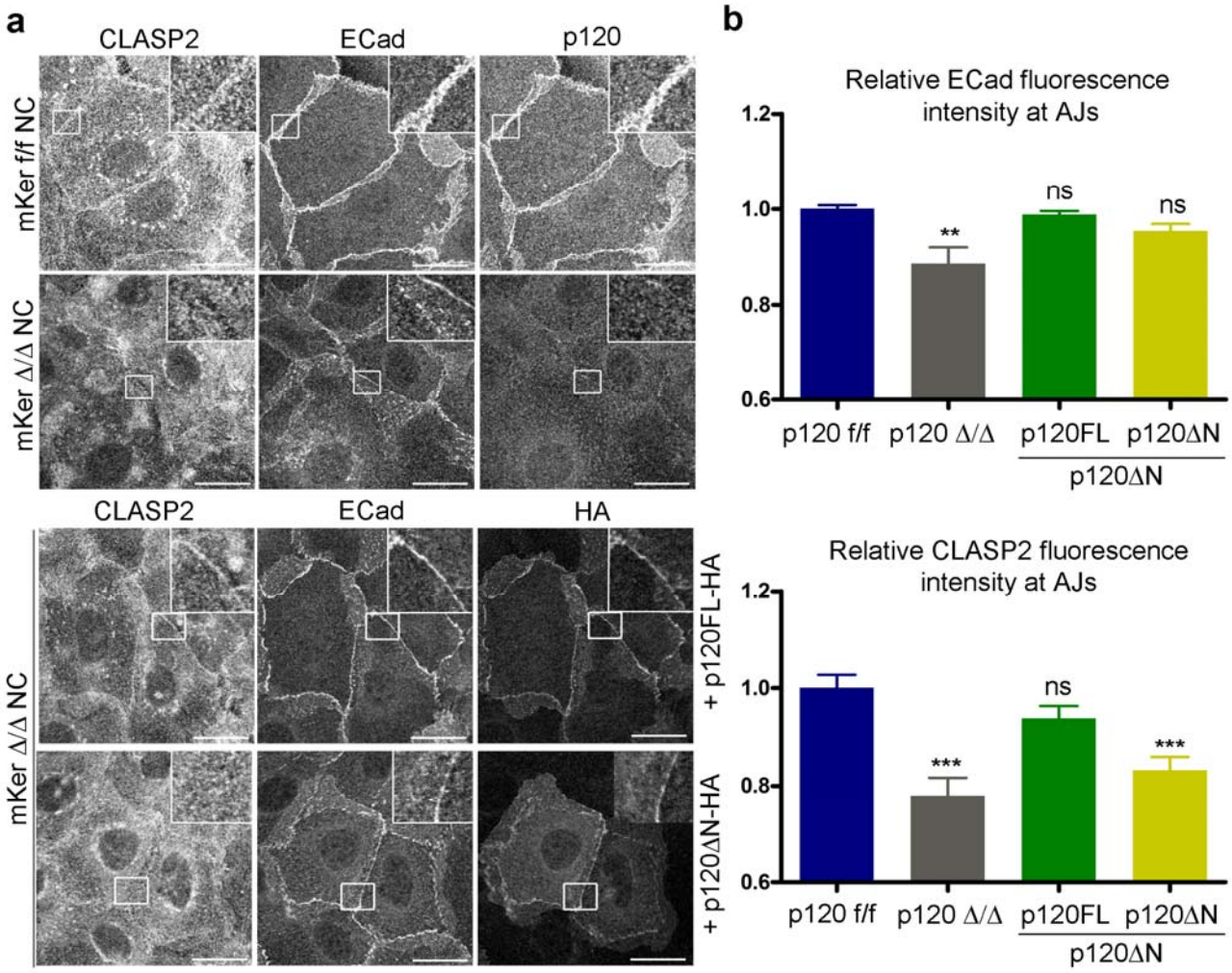


Figure 18 Re-expression of p120FL but not p120ΔN rescues CLASP2 localization to AJs. (a) p120-null mKer (p120 Δ/Δ) were transfected with either p120FL-HA or p120ΔN-HA. Non-transfected p120 f/f mKer were used as control. Cells were immunostained for ECad, CLASP2 and HA. Scale bar, 25 µm. **(b)** Quantification of ECad and CLASP2 fluorescence intensities at AJs. Random individual plot profiles (n = 10 per cell / 16 cells) were generated at sites of cell-cell adhesion. The maximum value of ECad fluorescence intensity and its associated CLASP2 fluorescence intensity were quantified. Data is normalized to control values (p120 f/f mKer) and represented as mean ± s.e.m; ** $p < 0.002$, Mann Whitney U test for ECad, *** $p < 0.0003$, Student's t test for CLASP2.

1.2.3. The p120 N-terminal domain is required to properly localize CLASP2 to Adherens Junctions

The determination of the domains responsible for the CLASP2-p120 interaction allowed us to perform rescue experiments. To test whether the lack of CLASP2 recruitment to AJs in p120-null

cells was a direct consequence of p120 deficiency, we re-expressed p120 in our p120 Δ/Δ mKer. Re-expression of p120 not only rescued ECad levels at the membrane, but also CLASP2 localization to AJs (Fig. 18 a, b). Importantly, a mutant version of p120 lacking the N-terminal domain was able to rescue ECad levels at the membrane but not CLASP2 localization to AJs (Fig. 18 a, b). Thus, the N-terminal domain of p120 is responsible for the CLASP2 localization at AJs.

2. Determine the role of CLASP2 at Adherens Junctions

2.1. Consequences of CLASP2 deficiency at the level of Adherens Junctions

2.1.1. CLASP2 deficiency leads to a delay in Adherens Junctions formation

We learned from the results obtained in our first objective that CLASP2 and p120 directly interact at AJs, and that p120 is responsible for localizing CLASP2 to AJs. Given the fact that CLASP2 is enriched at AJs, what role does it play in the formation/stability/turnover of AJs? And what is the possible mechanism for this possible uncovered role?

To answer these questions we knocked-down the expression of CLASP2 in mKer using a specific shRNA expressed in a lentiviral vector. The efficiency of the knock-down was evaluated by RT-PCR using primers specific for the different CLASP2 isoforms. This approach also allowed us to determine that only the long CLASP2α isoform is expressed in mKer (data not shown). For this reason, all mRNA analyses of CLASP2 were done using primers for total CLASP2 as well as primers specific for the α-isoform. The knock-down was very efficient, as observed by RT-PCR (Fig. 19a), WB (Fig. 19b) and immunofluorescence (Fig. 19c). Importantly, CLASP1 levels did not change after knocking-down CLASP2 (Fig. 19a, b).

The total levels of AJs proteins did not present overall changes in the absence of CLASP2, neither in low calcium conditions nor upon AJs formation with calcium (Fig. 19d). Thus, AJs components are properly expressed in absence of CLASP2, but do they properly localize to the membrane upon calcium switch to form cell-cell contacts? To answer this question we analyzed AJs formation by immunofluorescence in CLASP2 deficient (CLASP2 KD) and Scramble control mKer.

Scramble and CLASP2 deficient mKer were switched to a calcium containing media to induce AJs formation and the localization of p120 was evaluated at different time points of the calcium switch. We observed a significant decrease in the levels of p120 at the membrane at different stages of contact formation (Fig. 20a, b). This phenotype was more noticeable after 6 and 12 hours of calcium addition (Fig. 20b), suggesting that CLASP2 may be necessary to concentrate AJs components at the membrane. Analysis of ECad and α-catenin levels at the membrane showed equivalent results (Fig. 21a, b). We further validated these observations by analyzing the levels of AJs components on lysates of purified surface proteins. Control mKer gradually increased the levels of AJs components at the membrane upon addition of calcium to the medium, yet this gradual increase of AJs components was not observed in CLASP2 deficient mKer (Fig. 21c). Moreover, CLASP2 deficient mKer already displayed certain levels of ECad, p120 and α-catenin in low calcium conditions,

probably reflecting the fact that these cells are already differentiated in absence of calcium, as will be described later on in the text. Thus, CLASP2 deficiency leads to altered AJs formation and deficient concentration of AJs components at the membrane.

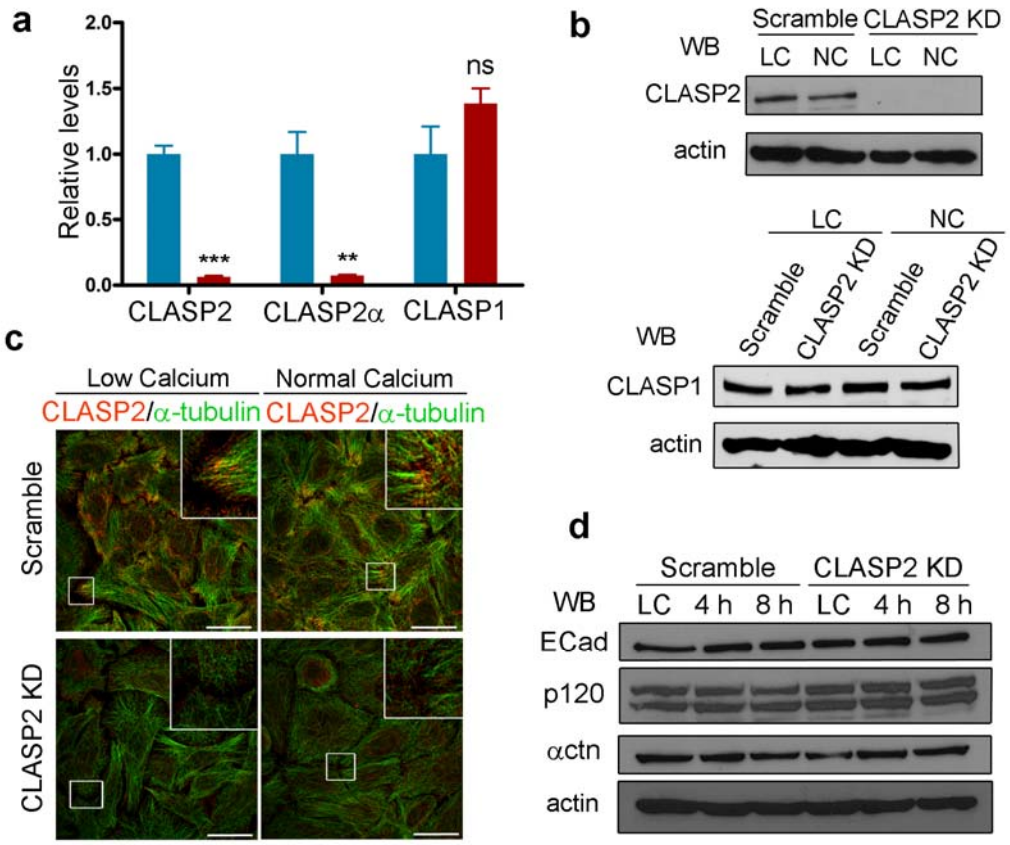


Figure 19 CLASP2 deficiency in mKer does not lead to changes in the total levels of AJs proteins. (a) RT-PCR analysis showing the levels of total CLASP2, CLASP2α and CLASP1 in mKer infected with lentiviruses expressing either a Scramble shRNA control or a CLASP2 specific shRNA (CLASP2 KD) after selection with G418. Data is represented as mean ± s.e.m.; *** $p<0.0001$, * $p<0.002$, Student's t test. ns: non-significant. **(b)** Total proteins from Scramble and CLASP2 KD mKer in Low Calcium (LC) and Normal Calcium (NC) conditions immunoblotted for CLASP2 and CLASP1 with their corresponding actin loading control. **(c)** Scramble control and CLASP2-deficient mKer were immunostained for CLASP2 and α-tubulin in absence (LC) or presence (NC) of calcium. Scale bar, 25 μm. **(d)** Total proteins from Scramble and CLASP2 KD mKer were immunoblotted for AJs proteins: ECad, p120 and α-catenin (αctn), at different time points of a calcium switch time course experiment. h represents the number of hours mKer were cultured in the presence of calcium.

The observed effect in AJ formation could be due to a loss of interaction between ECad and p120 in absence of CLASP2. In other words, CLASP2 could be important to maintain the ECad-p120 interaction. If the ECad-p120 interaction is weakened, cadherins are not stable at the membrane and are rapidly internalized (Davis et al., 2003; Ireton et al., 2002; Nanes et al., 2012). To test this possibility we immunoprecipitated ECad from both Scramble control and CLASP2-deficient mKer at different time points of a calcium switch, but we could not detect any significant difference in the

levels of p120 co-immunoprecipitated with ECad (Fig. 22). Thus, CLASP2 deficiency does not lead to alterations in the binding of p120 to ECad.

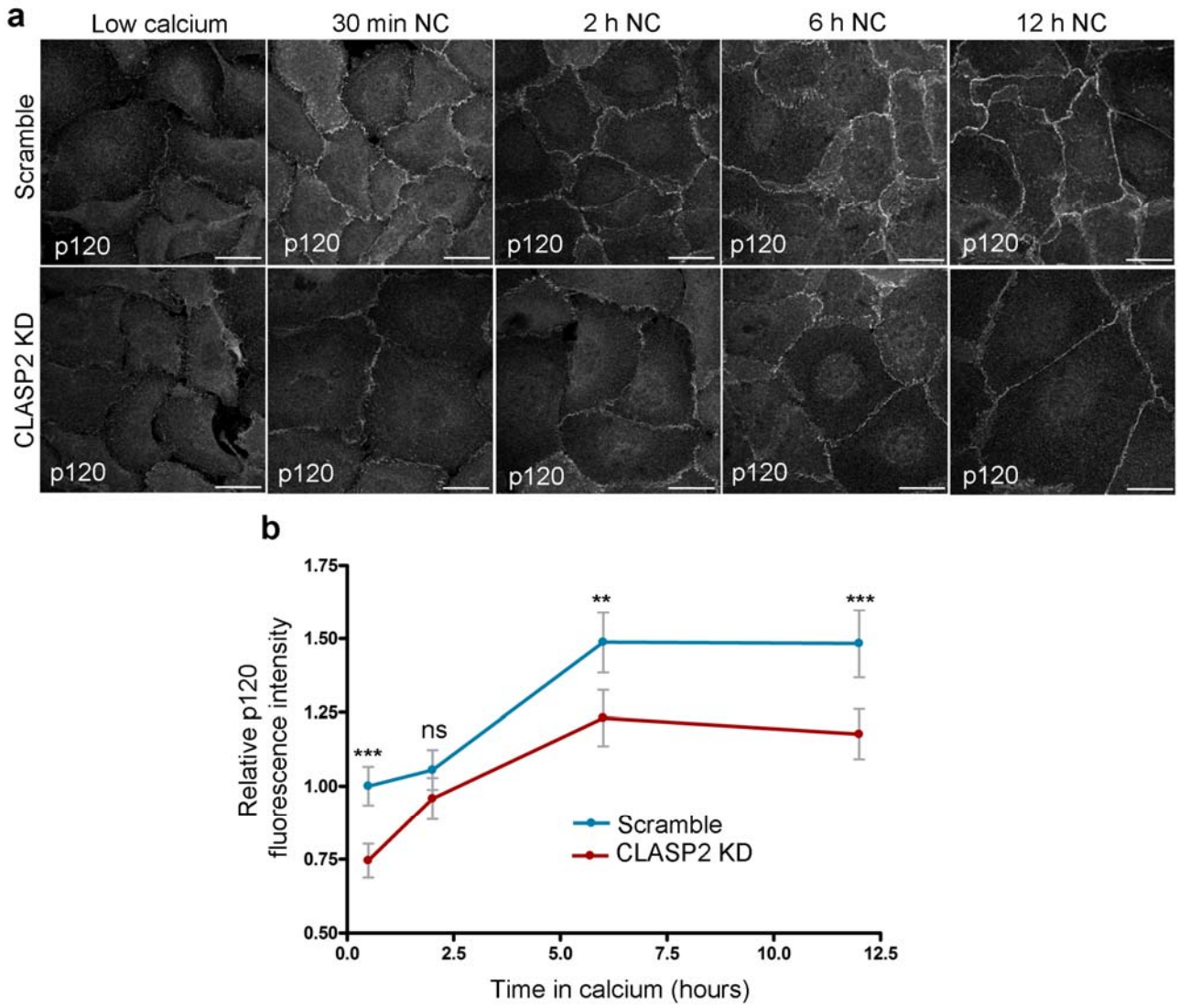


Figure 20 Absence of CLASP2 in mKer leads to impaired AJs formation. (a) Scramble control and CLASP2 deficient (CLASP2 KD) mKer were switched to a calcium containing media (NC: normal calcium) for different times points. Cells were immunostained for p120. Scale bar, 25 μm. **(b)** Quantification of p120 levels at the membrane in Scramble and CLASP2 deficient mKer. Random individual plot profiles were generated at sites of cell-cell adhesion (10 per cell) and the maximum fluorescence intensity was quantified. Data is represented as mean ± s.e.m.; ** $p < 0.005$, *** $p < 0.0005$, Student's t-test. ns: non-significant.

2.1.2. CLASP2 deficiency leads to altered Adherens Junctions dynamics

We observed that AJs formation was altered in absence of CLASP2. To have more quantitative information regarding the alterations in AJs dynamics in absence of CLASP2 we performed Fluorescence Recovery after Photobleaching (FRAP) experiments. This technique provides

quantitative data on the mobility of a fluorescent protein. A region of interest (ROI) is determined (in our case an area of cell-cell contact) and the fluorescence in this area is bleached. With time the fluorescence is recovered due to the arrival of new non-bleached molecules to the bleached region. The recovery of the fluorescence is followed by time-lapse microscopy. Stable molecules will recover slowly, whereas very dynamic molecules will show a fast recovery.

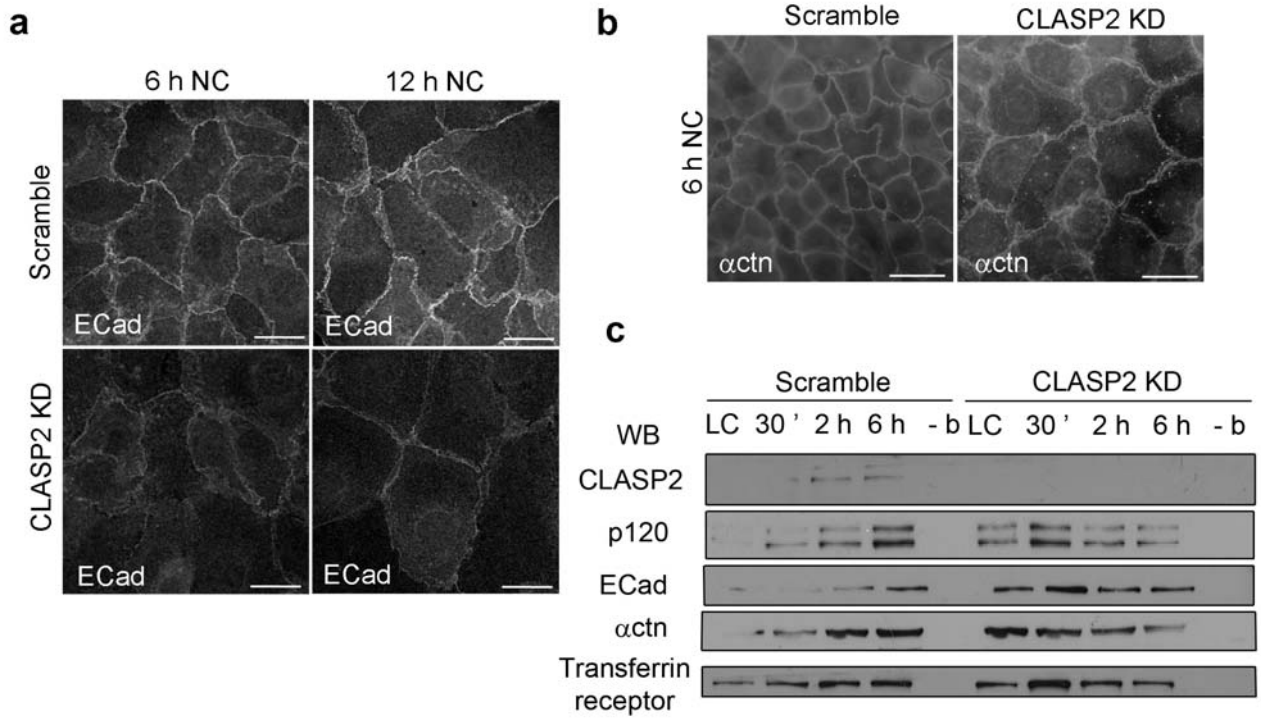


Figure 21 Absence of CLASP2 in mKer leads to impaired AJs formation. (a) Scramble and CLASP2 deficient mKer treated with calcium for 6 or 12 hours and immunostained for ECad. Scale bar, 25 μm. **(b)** Scramble and CLASP2 deficient mKer treated with calcium for 6 hours and immunostained for α-catenin (αctn). Scale bar, 50 μm. **(c)** Scramble and CLASP2 KD mKer were subjected to a calcium switch time course experiment. Cell surface proteins from the different time points were purified with biotin and streptavidin. Western Blot of the purified proteins was carried out and CLASP2, p120, ECad and αctn were analyzed. Transferrin receptor was used as a loading control.

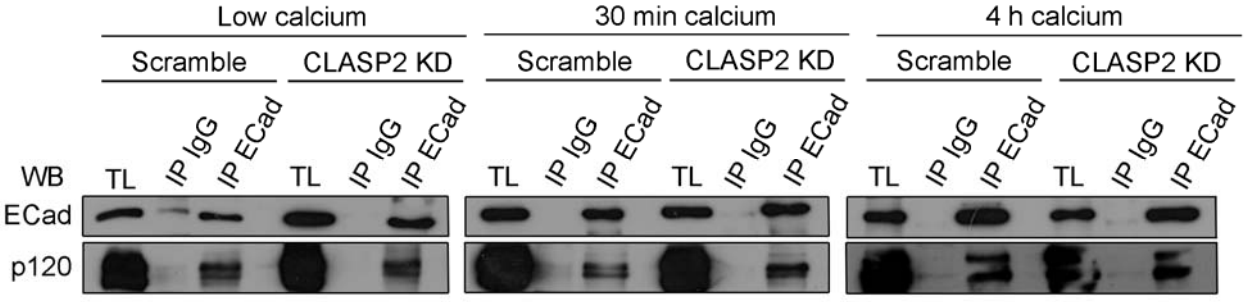


Figure 22 CLASP2 deficiency does not alter the binding of p120 to ECad. Scramble control and CLASP2-deficient (CLASP2 KD) mKer were immunoprecipitated for ECad at different time points of a calcium switch. The immunoprecipitates (IP) were blotted for ECad and p120. TL: total lysate.

We expressed p120-cherry in our Scramble control and CLASP2-deficient mKer and allowed the formation of AJs by adding calcium to the medium. Membrane regions with a clear enrichment of p120 and similar levels of fluorescence intensity were bleached and the recovery of the fluorescence was analyzed. The raw fluorescence recovery data was normalized to pre-bleach values and post-bleach values as described in the materials and methods section. From this analysis we obtained two different parameters: the half-life of p120 at AJs, meaning the time required to reach half of the plateau fluorescence intensity, and the mobile fraction, in other words, the p120-cherry fraction that is subjected to recycling. Both parameters were significantly decreased in absence of CLASP2, which means the global recovery of p120-cherry fluorescence is lower in CLASP2-deficient mKer (Fig. 23). We can envision two different scenarios to explain this result: in

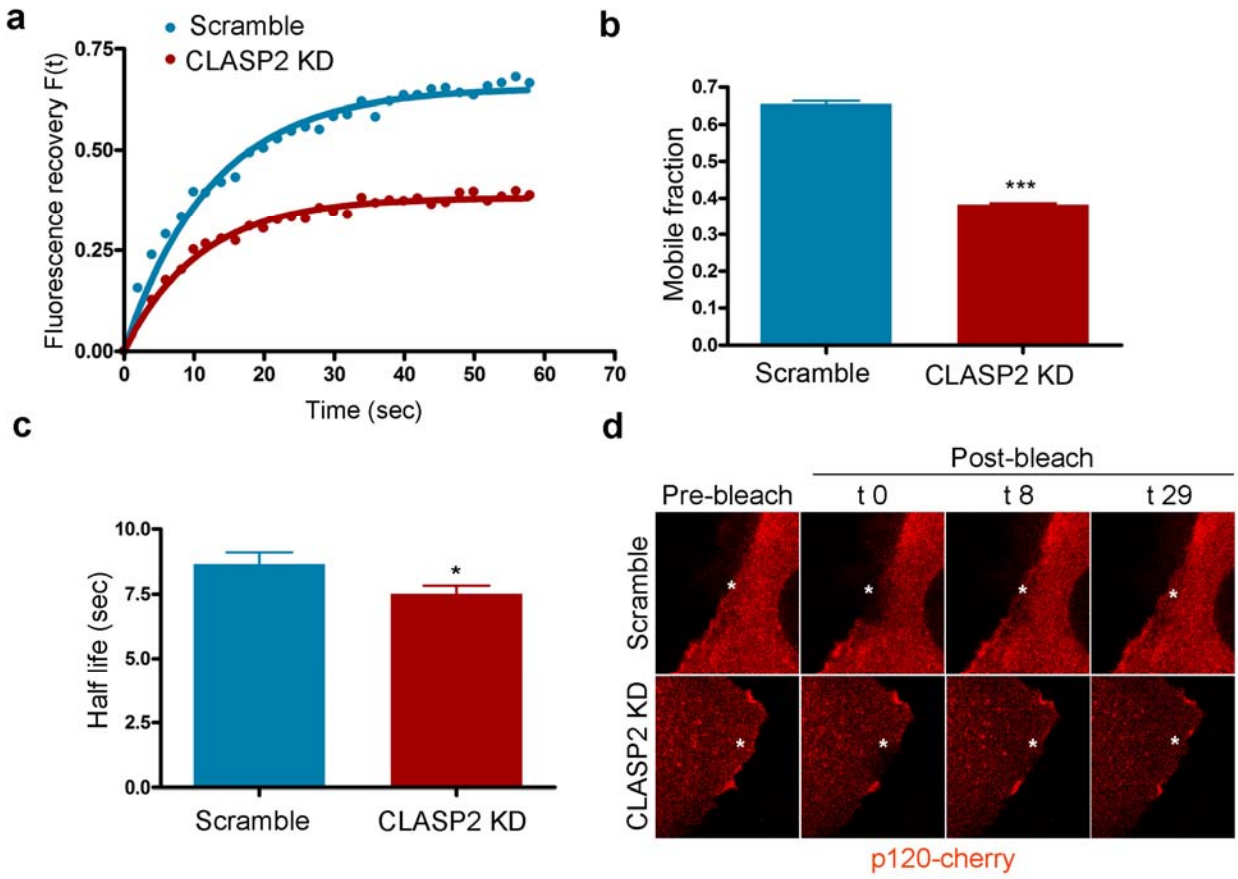


Figure 23 CLASP2 deficiency leads to a decrease in p120 dynamics at cell-cell contacts (a) Graph showing the fluorescence recovery after photobleaching of p120-cherry in a ROI selected at an area of cell-cell contact, in both Scramble and CLASP2 KD mKer. The vertical lines represent means ± s.e.m. and solid lines are best-fit single exponential curves. The data was normalized to pre-bleach and post-bleach values. **(b)** p120-cherry mobile fraction in Scramble and CLASP2 KD mKer (n = 13 cells, 4 independent experiments). Data is represented as mean ± s.e.m.; *** $p < 0.0001$, Student's t-test. **(c)** p120-cherry half life in Scramble and CLASP2 KD mKer. Data is represented as mean ± s.e.m.; * $p < 0.05$, Student's t-test. **(d)** Representative time-frames of the FRAP experiment from Scramble and CLASP2 KD mKer showing p120-cherry before the bleaching and at different time points after the bleaching. The asterik indicates the ROI.

absence of CLASP2 there are p120 molecules that do not move and therefore cannot be exchanged with non-bleached molecules (a problem of recycling), or alternatively, the arrival of new p120 molecules to the bleached region is reduced (a problem of delivery). Since nocodazole treatment does not impair the traffic of cadherins to the membrane, but affects cadherin internalization, we hypothesize that CLASP2 plays a major role in the maintenance of AJs. Further experiments using MT depolymerizing drugs in absence of CLASP2 will be needed to clarify whether CLASP2 plays a role in the delivery of cadherins to the membrane.

Previous studies have shown that treatment with low doses of nocodazole, which is known to inhibit MT plus-end dynamics, leads to a decrease in the mobility of cadherins at cell-cell junctions (Stehbens et al., 2006). Equivalent results were obtained with a Clip170 mutant unable to localize at the cortex. We therefore reasoned that the underlying mechanism responsible for the decreased dynamics of junctional components could be rooted to alterations in the MT network.

2.2. Mechanism of action of CLASP2 in the maintenance of proper Adherens Junctions dynamics

2.2.1. CLASP2 and p120 deficiency lead to a decrease in microtubule targeting to Adherens Junctions

We hypothesized that the decrease in p120-cherry dynamics in absence of CLASP2 was due to a decrease in MT targeting to AJs. Such a decrease in MT targeting could lead to alterations in the recycling/internalization of p120 molecules. To evaluate this possibility we counted the number of MTs that reached cadherin-based cell-cell contacts in Scramble and CLASP2KD cells after calcium treatment. By visual inspection it was already evident that CLASP2 deficiency led to a decrease in the density of cortical MTs (Fig. 24a). Quantification of the number of MTs that specifically reached an area of cadherin-based cell-cell adhesion showed that absence of CLASP2 led to a significant decrease in MT targeting to AJs (Fig. 24b).

Although we cannot conclude that this decrease in MT targeting to AJs is a consequence of an altered p120-CLASP2 interaction, it was interesting to observe that p120 deficiency led to an equivalent phenotype in terms of the number of MTs that reached an area of cell-cell contact (Fig. 24a, b). This result suggests that both proteins may function coordinately to regulate MT targeting to AJs.

2.2.2. CLASP2 deficiency in mouse keratinocytes leads to a decrease in microtubule stability

As mentioned in the introduction, absence of either CLASP2 or p120 have been correlated with a decrease in MT stability (Drabek et al., 2012; Drabek et al., 2006; Ichii and Takeichi, 2007; Schmidt et al., 2012). It is also known that cell-cell contact formation leads to MT stabilization (Chausovsky et al., 2000; Waterman-Storer et al., 2000). Therefore, we decided to evaluate whether in our system cell-cell contact formation triggered MT stabilization, and, most importantly, if this process was altered in absence of CLASP2 or p120.

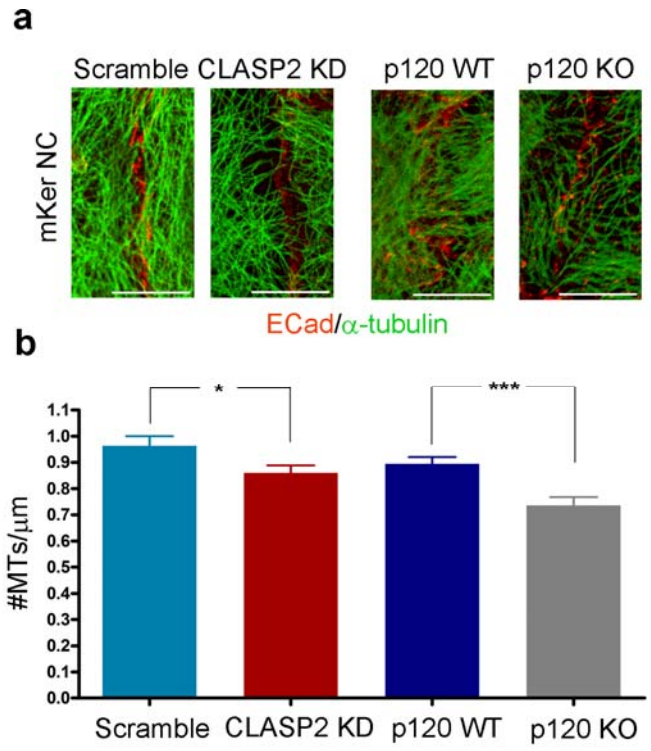


Figure 24 CLASP2 deficiency and p120 deficiency lead to a decrease in the number of MTs that reach cadherin-based cell-cell adhesions. (a) CLASP2-deficient and p120-null mKer with their corresponding Scramble and p120 f/f controls stained for ECad and α-tubulin. Scale bar, 25 µm. **(b)** Quantification of the number of microtubules that reach an area of cell-cell contact. Only regions with well-formed AJs were selected for the analysis. (n = 60 cells, 2 independent experiments). Data is represented as mean ± s.e.m.; * $p < 0.04$, *** $p < 0.0005$, Student's t-test.

MT resistance to cold treatment has been classically used as a way to evaluate MT stability. However, MTs in mKer seem to be resistant to cold, since even after 4 hours of cold treatment the MT network was intact (data not shown). We also analyzed bona-fide post-translational modifications of tubulin associated to increased MT stability. Surprisingly, we could not detect detyrosinated tubulin (glu-tubulin) in mKer, unless cells were pre-treated with taxol (Fig. 25a). In contrast, acetylated-tubulin (ace-tubulin) was detected by WB but its levels did not change upon cell-cell contact formation, neither in absence of p120 nor CLASP2 (Fig. 25a).

We further used MT resistance to nocodazole as a gold standard treatment to assess MT stability. mKer with well-formed AJs were treated with 5µM nocodazole for 30 min following extraction of the monomeric tubulin to allow visualization of individual resistant MTs. The nocodazole treatment

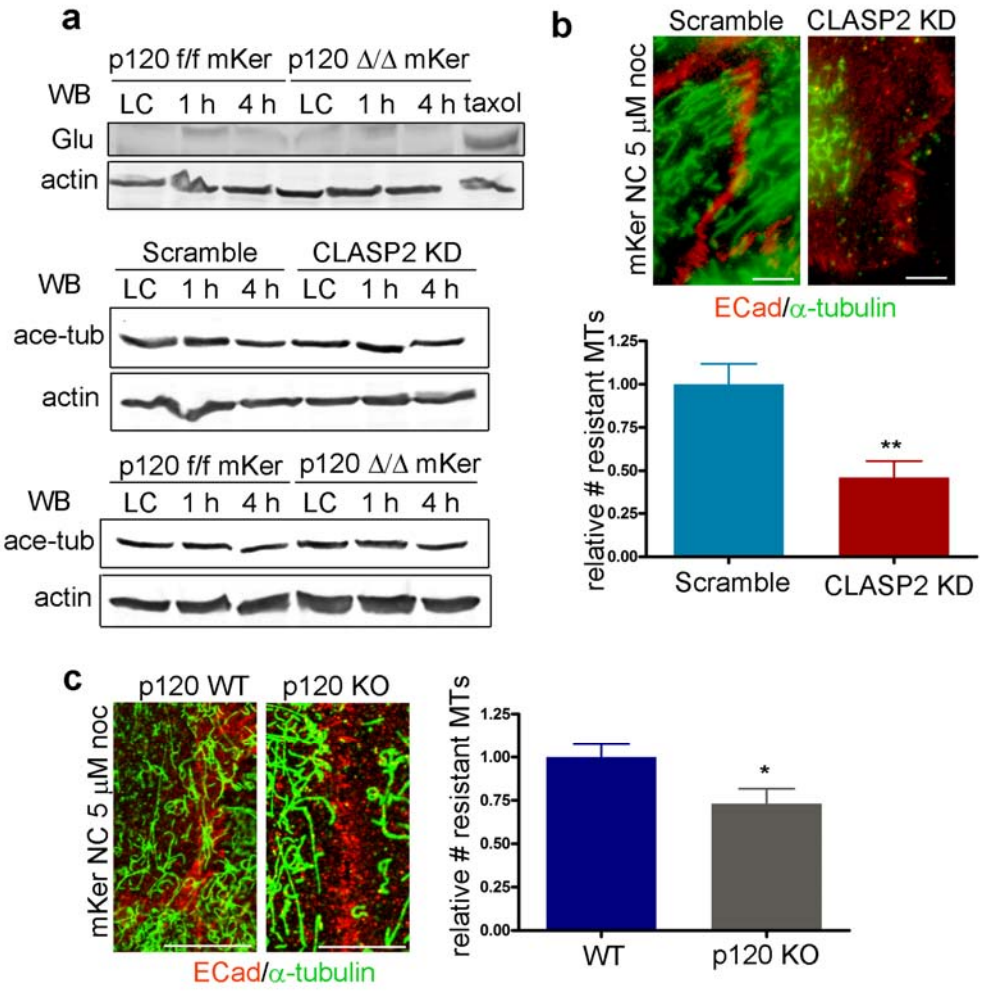


Figure 25 Absence of either p120 or CLASP2 leads to a decrease in the number of stable MTs at AJs without global changes in acetylated and detyrosinated tubulin. (a) Total levels of acetylated tubulin (ace-tub) and detyrosinated tubulin (Glu) evaluated by Western Blot in Scramble control, CLASP2-deficient (CLASP2 KD), p120 control (p120 f/f) and p120-null (p120 D/D) mKer. Cells were grown in low calcium (LC) or switched to a calcium containing medium for 1 and 4 hours. mKer treated with taxol were used as a positive control of detyrosinated tubulin. **(b)** Scramble and CLASP2-deficient mKer were grown with calcium for 4 hours following addition of 5 mM nocodazole for 30 minutes and extraction of monomeric tubulin. Cells were stained for ECad and α-tubulin. Scale bar, 25 μm. The number of MTs present at AJs was counted. Only regions with well-formed AJs were selected for the analysis. (n = 30 cells). Data is normalized to control values and represented as mean ± s.e.m.; ** $p < 0.002$, Student's t-test. **(c)** WT and p120 KO mKer were grown with calcium for 4 hours following addition of 5 mM nocodazole for 30 minutes and extraction of monomeric tubulin. Cells were stained for ECad and α-tubulin. Scale bar, 12.5 μm. The number of MTs present at AJs was counted. (n = 30 cells). Data is normalized to control values and represented as mean ± s.e.m.; * $p < 0.05$, Student's t-test.

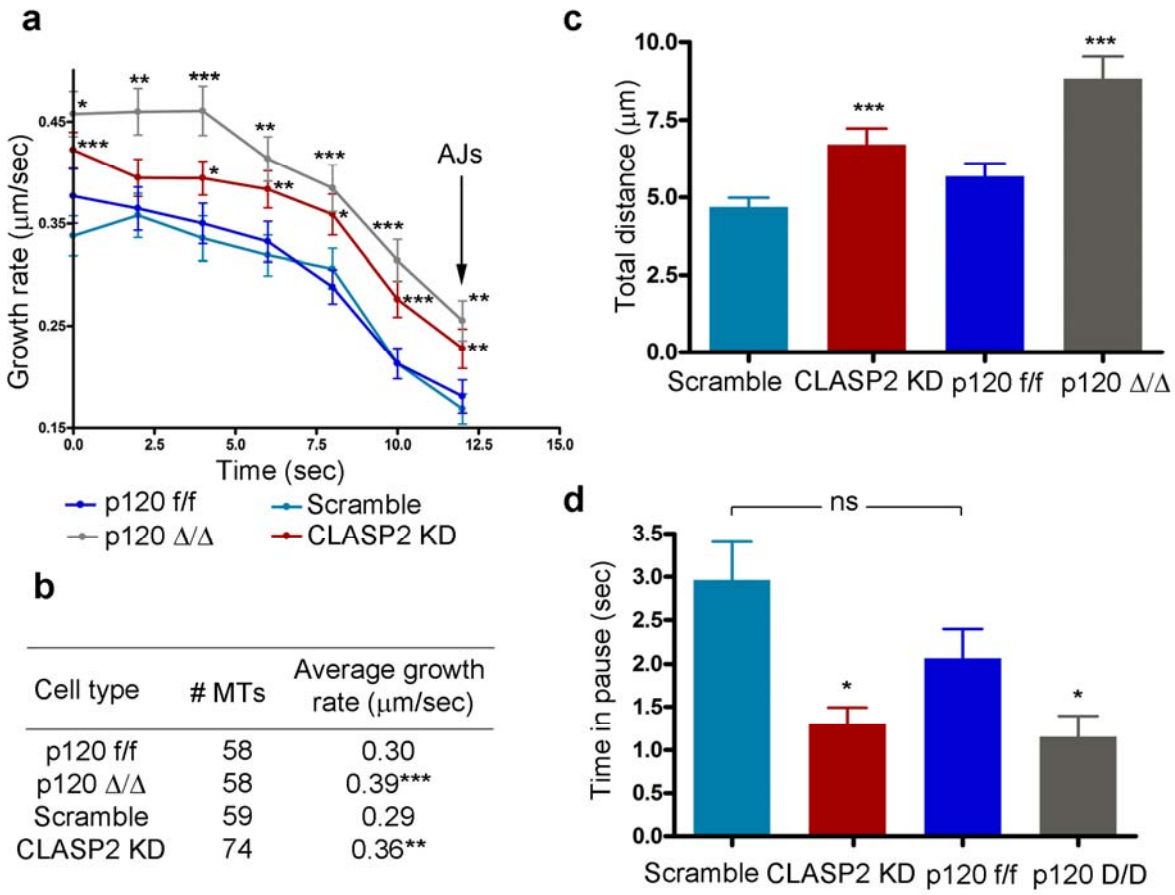
conditions (amount and duration of the treatment) were empirically determined as those that allowed visualization of individual MTs without depolymerizing the whole network (data not shown). Both p120-null and CLASP2-deficient mKer showed a significant decrease in the number of MTs resistant to the treatment that localized specifically to AJs when compared to controls (Fig. 25b, c). This decrease was more dramatic in cells lacking CLASP2 (Fig. 25c). Whether the observed phenotype is a general feature of CLASP2-deficient and p120-null mKer or is specific to AJs remains to be further evaluated.

MT targeting to cell-cell adhesion sites is known to induce a decrease in MT growth rates and MT stabilization (Komarova et al., 2012). Our MT resistance to nocodazole data shows that absence of either CLASP2 or p120 leads to a decrease in MT stabilization at AJs, indicating a possible defect in MT targeting. To further validate this hypothesis, we analyzed MT dynamics at AJs in terms of speed and directionality.

2.2.3. Microtubule dynamics at Adherens Junctions are altered in absence of either CLASP2 or p120

To gain further insight into how MTs reach AJs in absence of CLASP2 we analyzed the MT network by stably expressing via retroviruses cherry-tubulin in mKer. However, due to the high density of MTs at sites of cell-cell adhesion we were unable to follow the ends of individual MTs. For this reason, we used the bona-fide MT +TIP protein EB3 to track the ends of MTs (Stepanova et al., 2003). This approach has been widely used in the field to analyze MT dynamics. In particular, it has been shown that EB1 comets target gap junctions and stay in their vicinity for longer times when compared with cell free edges (Shaw et al., 2007). Analysis of EB3-YFP dynamics at VECad-adhesion sites has also shown that MT growth rates are reduced upon contact formation (Komarova et al., 2012). However, this approach has a clear disadvantage: as discussed in the introduction, EBs promote growth and stabilization of MTs (Morrison, 2007), and therefore they increase the real growth rate of MTs and cannot be used to determine shrinkage rates or track long events of pausing or catastrophes.

Briefly, both Scramble control and CLASP2-deficient mKer were transfected with a construct encoding EB3-GFP. Time-lapse images were taken every 2 seconds and individual EB3-GFP comets were manually tracked at areas of cell-cell contact (visualized by DIC microscopy). We focused our analysis on the last 12 seconds before the EB3 signal fell off the MT upon reaching an area of cellcell contact, which could represent either a pausing event or a MT catastrophe. This type of analysis enabled us to measure MT growth rate, and thus we could determine that EB3 comets in CLASP2-deficient cells presented a higher growth rate during the whole course of the analysis (Fig. 26a, b). This may reflect an inability of CLASP2-deficient MTs to properly pause at AJs to be captured and stabilized. Interestingly, when this analysis was done in p120-null mKer equivalent results were found: absence of p120 leads to an increase in the growth rate of EB3 comets upon reaching areas of cell-cell contact, as shown by measuring the speed in each frame (Fig. 26a) and the average speed during the 12 seconds analyzed (Fig. 26b).



b

Cell type	# MTs	Average growth rate (µm/sec)
p120 f/f	58	0.30
p120 Δ/Δ	58	0.39***
Scramble	59	0.29
CLASP2 KD	74	0.36**

Figure 26 EB3 comets present higher growth rates and decreased pausing times at AJs in p120-null and CLASP2-deficient mKer. (a) EB3-GFP was expressed in Scramble control cells, CLASP2-deficient cells (CLASP2 KD), p120 control cells (p120 f/f) and p120-null cells (p120 Δ/Δ). Time-lapse images were taken every 2 seconds. Individual EB3-GFP comets were manually tracked during the 12 last seconds before reaching an area of cell-cell contact. The speed in each frame is shown. (n = 10 cells, p120 f/f: 58 microtubules, p120 Δ/Δ: 58 microtubules, Scramble: 59 microtubules, CLASP2 KD: 74 microtubules). Data is represented as mean ± s.e.m.; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.04$, Student's t test. **(b)** Average EB3 speed during the 12 seconds analyzed in panel a in the 4 cell types indicated; *** $p < 0.0001$, ** $p < 0.003$, Student's t test. **(c)** The distance covered by individual EB3-GFP comets in the XY plane during the time analyzed was quantified. Data is represented as mean ± s.e.m.; *** $p<0.001$. Mann Whitney U test. **(d)** Quantification of the time during which individual EB3-GFP comets did not move more than 0.5 µm from one frame to the next. This value was set as the reference point to define pausing microtubules. Data is represented as mean ± s.e.m.; * $p<0.05$. Mann Whitney U test.ns: not signicant.

Reflecting the fact that MTs grow faster in the vicinity of AJs both in p120-null and CLASP2-deficient cells, the total distance covered by individual MTs during the 12 seconds analyzed was also significantly higher when compared to controls (Fig. 26c). Changes in MT length of less than 0.5 µm between two consecutive frames are considered as pause (due to the limits imposed by the microscopy technique itself) (Kamath et al.). We used this criterion to analyze the time MTs expend in pause, although since the tracking was done by overexpressing EB3, the numbers obtained do not represent the real value of the time the MT spent in pause. Using this threshold we could see that the average time spent in pause was double in control cells compared to the corresponding

p120-null and CLASP2-deficient mKer (Fig. 26d), further supporting the idea that MTs do not pause and thus, are not stabilized at cell-cell adhesion sites.

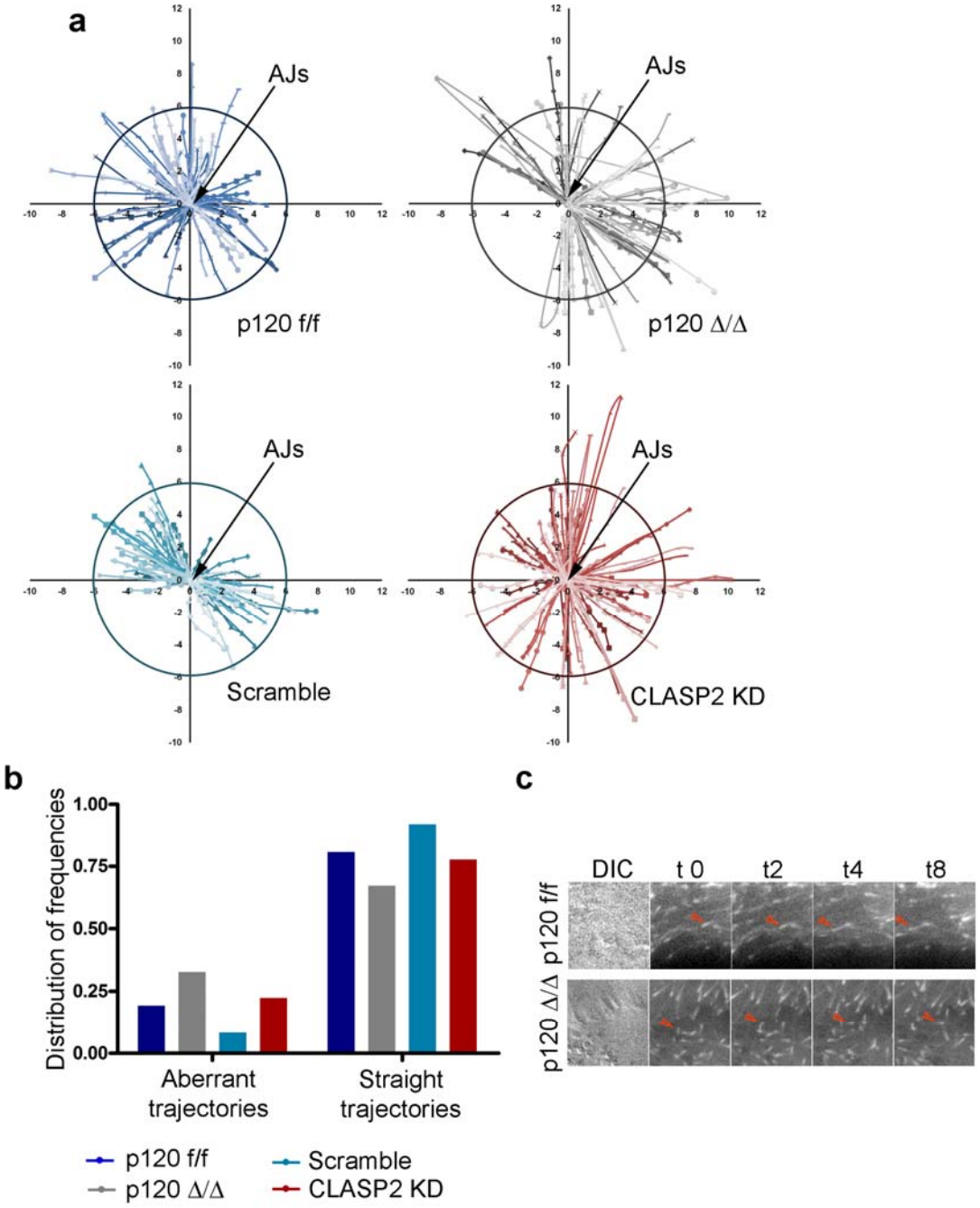


Figure 27 EB3-GFP comets present random trajectories at AJs in p120-null and CLASP2-deficient mKer. (a) Individual trajectories of EB3-GFP comets in the XY plane were normalized to the last point of the trajectory, corresponding to the moment in which the EB3-GFP comet reaches the junction and falls off the microtubule. Individual frames were taken every 2 seconds and the data corresponds to the last 12 seconds before the comet reaches the junction. **(b)** Frequency distribution of microtubules showing aberrant trajectories or straight trajectories from panel a. **(c)** Individual time-lapse frames showing examples of EB3-GFP comets (arrowheads) reaching an area of cell-cell contact in control (p120 f/f) and p120-null (p120 Δ/Δ) mKer. Note that in p120 Δ/Δ mKer the EB3 comet continues growing parallel to the region of cell-cell contact. t: time in seconds.

From the analysis of the EB3-GFP time-lapse experiments we also obtained data regarding MT plus-end position at each time point. To visualize MT trajectories, we normalized each coordinate in the XY plane to the last point of the trajectory, which corresponds to the moment in which the EB3 comet reaches an area of cell-cell contact. EB3 comets in CLASP2-deficient and p120-null mKer not only covered longer distances during the same period of time, as mentioned above, but also presented more aberrant trajectories and did not reach the cell-cell contacts following a straight path when compared with the corresponding controls (Fig. 27a). Additionally, in the case of p120-null cells, we observed that many MTs continued growing and did not stop at cell-cell contacts (Fig. 27b). This reflects an improper MT targeting to cortical structures and has been described for models of loss-of-function of other MT binding proteins, such as Acf7 in the context of MT dynamics at focal adhesions (Kodama et al., 2003), and EB in the context of VECad (Komarova et al., 2012). In the case of CLASP2-deficient mKer we did not observe MTs bending and growing beyond areas of cell-cell contacts, although more than 70 MTs were followed, possibly due to the increased catastrophe and MT shortening associated to CLASP2-deficiency (Mimori-Kiyosue et al., 2005).

In summary, absence of either p120 or CLASP2 leads to a decrease in MT targeting to AJs as measured by counting the numbers of MTs that reach cell-cell contacts, evaluating the speed of MTs near cell-cell adhesion sites and analyzing the trajectories of MTs upon reaching cell-cell contacts. The increased growth rate and the decreased MT stability at AJs can be used as readouts of MT targeting to AJs. Thus, absence of either CLASP2 or p120 leads to a decrease in MT targeting to cell-cell adhesion sites.

2.3. Physiological effects of CLASP2 deficiency at AJs in mKer

So far we have seen that CLASP2 and p120 directly interact at AJs and form a complex together with ECad. p120 deficiency compromises CLASP2 recruitment to AJs, and CLASP2 deficiency leads to altered AJs formation and dynamics. This phenotype may be a consequence of altered MT dynamics and decreased MT targeting to AJs. But, what are the consequences of such alterations for the normal physiology of AJs? In other words, what are the consequences for mKer to have altered AJs dynamics as a consequence of CLASP2 deficiency?

To answer this question we first evaluated AJs resistance to calcium removal in control and CLASP2-deficient mKer. As mentioned in the introduction, cadherins require calcium to mediate homophilic interactions between cells, and calcium removal causes AJs disassembly and ECad internalization (Kartenbeck et al., 1991; Niessen et al., 2011). We first induced cell-cell contact formation maintaining the cells in calcium-containing media for 6 hours, and then removed calcium and analyzed AJs at different time points. 6 hours after calcium treatment Scramble control cells

presented a clear CLASP2 enrichment at well-formed AJs and CLASP2-deficient cells had also formed AJs (Fig. 28a). 30 minutes of calcium deprivation were sufficient to start observing a clear decrease in the localization of ECad at the cell membrane in control cells, whereas CLASP2-deficient mKer already showed areas of cell-cell detachment (although positive patches of ECad could still be detected) (Fig. 28a). These observations suggest that absence of CLASP2 leads to a reduction in the strength of adhesion mediated by AJs. It was also interesting to observe that in control cells, upon calcium removal, CLASP2 staining pattern changed to a more MT plus-end tracking, enriched at certain membrane regions of the cells, which may be areas subjected to tension as a consequence of cells trying to hold each other together (Fig. 28a).

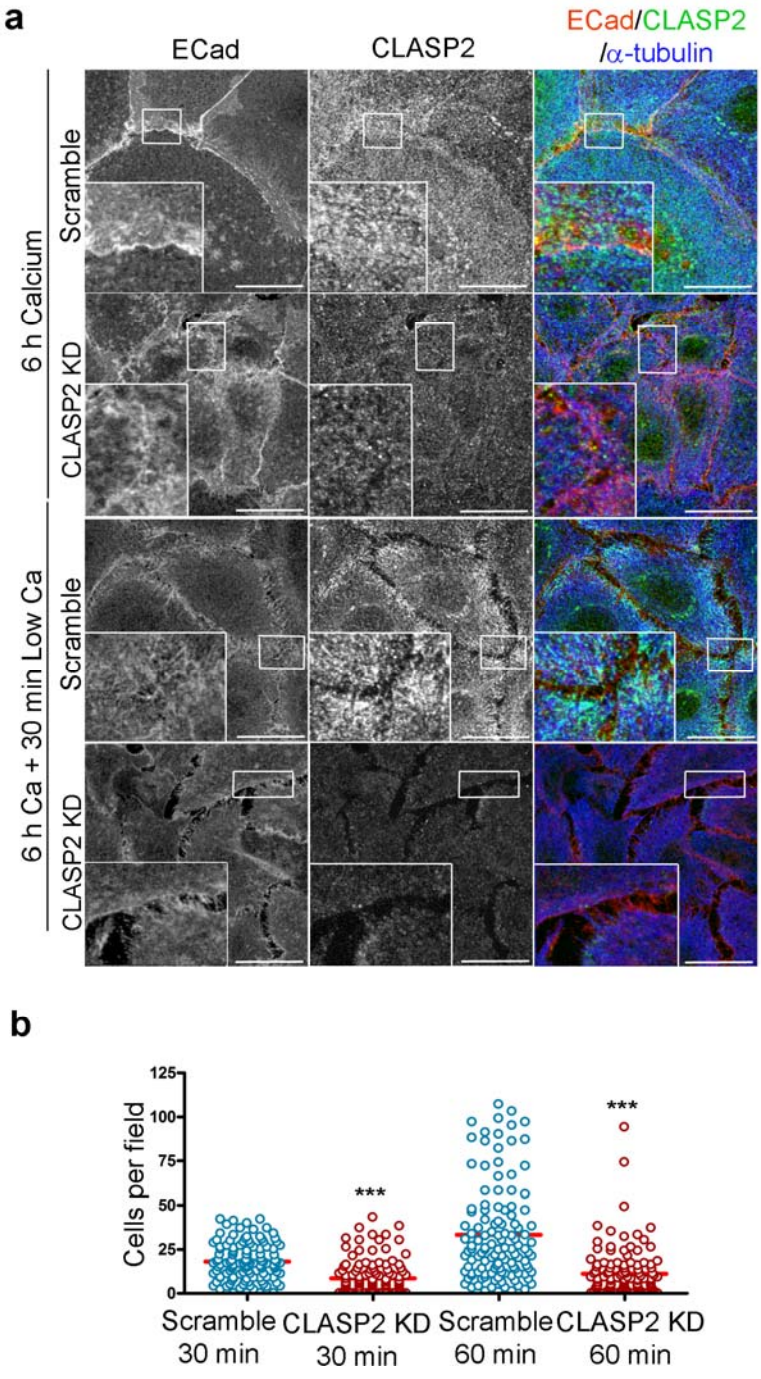


Figure 28 CLASP2 deficiency leads to the formation of less robust AJs when compared to WT mKer. (a) CLASP2 deficient mKer and their WT counterparts (Scramble) were treated for 6 hours with calcium to induce AJs formation. Calcium was removed from the cell culture media to induce AJs disassembly and 30 min after, cells were fixed and immunostained for ECad, CLASP2 and α-tubulin. Scale bar, 25 μm. **(b)** CLASP2 deficient mKer and their WT counterparts (Scramble) were allowed to attach to ECad-Fc-coated plates for 30 min and 1 hour. The number of cells attached per field was counted (n = 3 independent experiments, 40 images per experiment). As negative controls plates coated with mouse Fc and mKer in the presence of EDTA were used. Data is represented as mean ± s.e.m.; *** $p < 0.0001$, Mann-Whitney U test.

As a second proof that AJs are weaker in absence of CLASP2 we evaluated the ability of cells to attach to ECad-coated plates. To this end we coated plates with the extracellular domain of murine ECad bound to Fc and assessed the ability of Scramble control and CLASP2-deficient mKer to attach for different time points. Cells were initially trypsinized using crystalline trypsin to ensure that cadherin molecules were retained at the surface. Two negative controls of the experiment were used: cells plated in the presence of EDTA and cells plated in plates covered with mouse Fc alone. Following this approach, we could observe that the number of CLASP2-deficient mKer attached to the plates was significantly lower for the two time points considered (Fig. 28b). Thus, CLASP2-deficient cells present weaker AJs when compared to controls.

We have seen that absence of CLASP2 promotes alterations in the dynamic behavior of AJs components, which lead to altered cadherin-based cell-cell adhesion. Alterations in a single molecule thus have an impact on the physiological behavior of a cellular structure. We wanted to go one step further and analyze additional CLASP2 functions required for the maintenance of proper epidermal homeostasis.

3. Investigate the consequences of the loss of CLASP2 in the proliferation/differentiation balance of keratinocytes

We learned from the first two aims that CLASP2 and p120 interact at AJs, regulating AJs stability and MT targeting. CLASP2 is necessary for the proper adhesive function of AJs, yet it is unknown whether this has an effect in keratinocyte behavior. To address this question, we first analyzed the effects of CLASP2-deficiency *in vitro* in terms of proliferation and differentiation.

3.1. CLASP2 deficiency leads to alterations in the proliferation vs. differentiation balance *in vitro*

3.1.1. CLASP2-deficiency in mouse keratinocytes leads to spindle alterations and decreased proliferative potential

As mentioned in the introduction, CLASP2 deficiency leads to spindle alterations and mitotic defects in HeLa cells, MEFs and Mouse Adult Fibroblasts (MAFs) (Mimori-Kiyosue et al., 2006; Pereira et al., 2006) due to destabilization of kinetochore-MT attachments (Maia et al., 2012). We analyzed whether this was also the case in mKer *in vitro*. To this end we synchronized mKer at G1/S with a double thymidine block and analyzed mitotic cells by immunofluorescence 5 hours after the release. Absence of CLASP2 led to multiple spindle abnormalities such as: multipolar spindles, disorganized spindles, collapsed spindles and spindles with decreased MT density (Fig. 29a). Interestingly, p120-null mKer show the same spindle alterations, but the mechanism is currently unknown (Perez-Moreno et al., 2008). It remains to be elucidated whether CLASP2 and p120 cooperatively play a role in spindle formation/maintenance.

Such spindle alterations in CLASP2-deficient mKer gave rise to cells with increased DNA content as observed by FACS analysis of the cell cycle (Fig. 29b). These cells could either be arrested at mitosis or have increased ploidy. Indeed, CLASP2-deficient mKer also presented a proliferation defect: analysis of cell numbers in a growth assay showed that mKer deficient for CLASP2 grew more slowly that their WT counterparts (Fig. 29c). This decrease in proliferation led to a decrease in the colony-formation capabilities of primary CLASP2-deficient mKer (Fig. 29d). Not only CLASP2-deficient mKer formed fewer colonies, but also these colonies were smaller and not well-compacted as in Scramble control cells. Cells in the colonies presented typical differentiated morphologies (Fig. 29d). The decrease in colony formation capabilities was not a consequence of increased apoptosis (Fig. 30a).

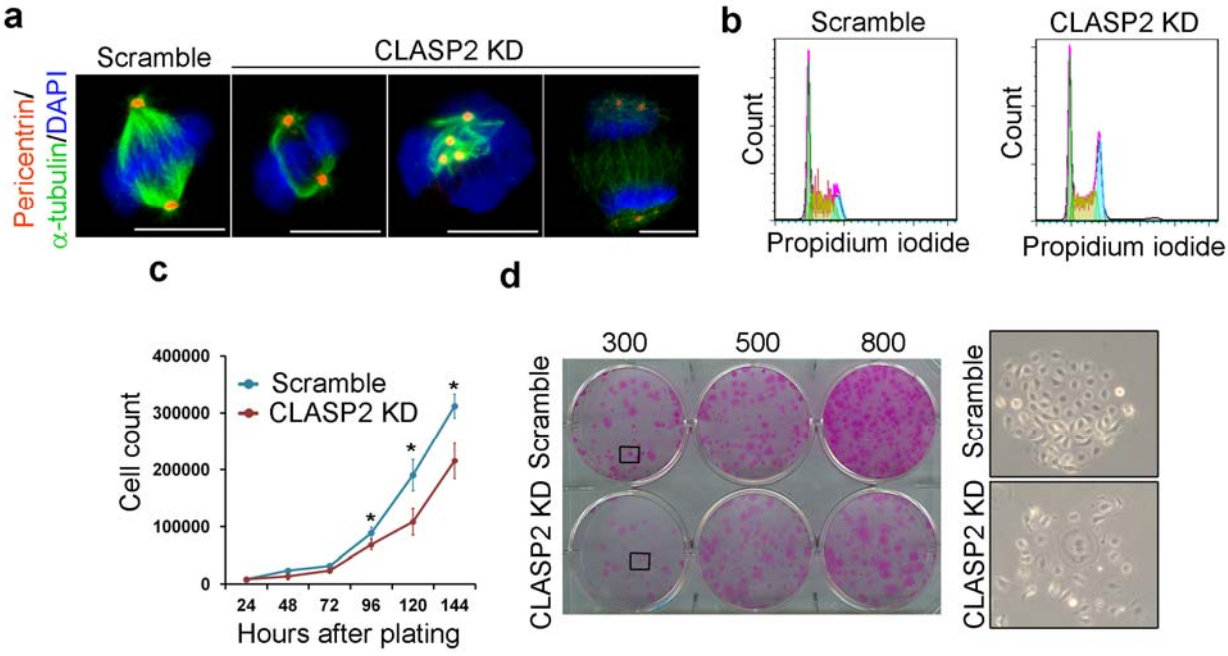


Figure 29 CLASP2-deficiency in mKer leads to spindle alterations and decreased proliferative potential. (a) Scramble control and CLASP2-deficient (CLASP2 KD) mKer were synchronized with a double thymide block and immunostained 5 hours after the release with pericentrin and α-tubulin antibodies. Scale bar, 10 μm. **(b)** Cell cycle profiles of Scramble and CLASP2 KD mKer stained with Propipdium Iodide. **(c)** Growth curves of Scramble and CLASP2 mKer. Equal numbers of cells were plated in triplicate for each time point. Cel numbers were counted every 24 hours. Data is represented as mean ± s.e.m.; * $p<0.05$, Student's t test. **(d)** Colony formation assay of Scramble control and CLASP2 KD mKer. Either 300, 500 or 800 cells were plated in fibronectin-coated plates, fixed and stained with crystal violet one week after plating. Images of representative colonies from both genotypes (black box) were taken using a brightfield microscope.

3.1.2. CLASP2-deficient mouse keratinocytes present hallmarks of differentiation

Upon differentiation *in vitro* epidermal keratinocytes increase in size (Sun and Green, 1976) and express differentiation markers of suprabasal layers such as Keratin 1 (K1), Keratin 10 (K10) or Filaggrin (Candi et al., 2005). Since the cells observed in our colony formation assays presented a morphology characteristic of differentiated cells, we explored this aspect with more detail.

First, we quantified the size of CLASP2-deficient mKer compared to Scramble controls and we detected a significant increase (Fig. 30b). Equivalent results have been observed upon depletion of CLASP2 in a cell line of human keratinocytes (HaCaT) (Torsten Wittmann, UCSF, US; personal communication) (note the difference in size in figure 20 and 21). Next, we evaluated by immunoblot the levels of the above mentioned differentiation markers. Strikingly, even at low calcium conditions in which mKer should remain undifferentiated and proliferative, we could see clear expression of K1, K10 and Filaggrin (Fig. 30c). Upon calcium treatment, differentiation markers

gradually increased in Scramble controls, but they were highly expressed in CLASP2-deficient mKer at all time points analyzed (Fig. 30c). These results were also validated by RT-PCR (data not shown).

Therefore, CLASP2-deficiency in primary mKer *in vitro* leads globally to a loss of basal progenitor characteristics, in terms of a decrease in proliferation and an increase in differentiation.

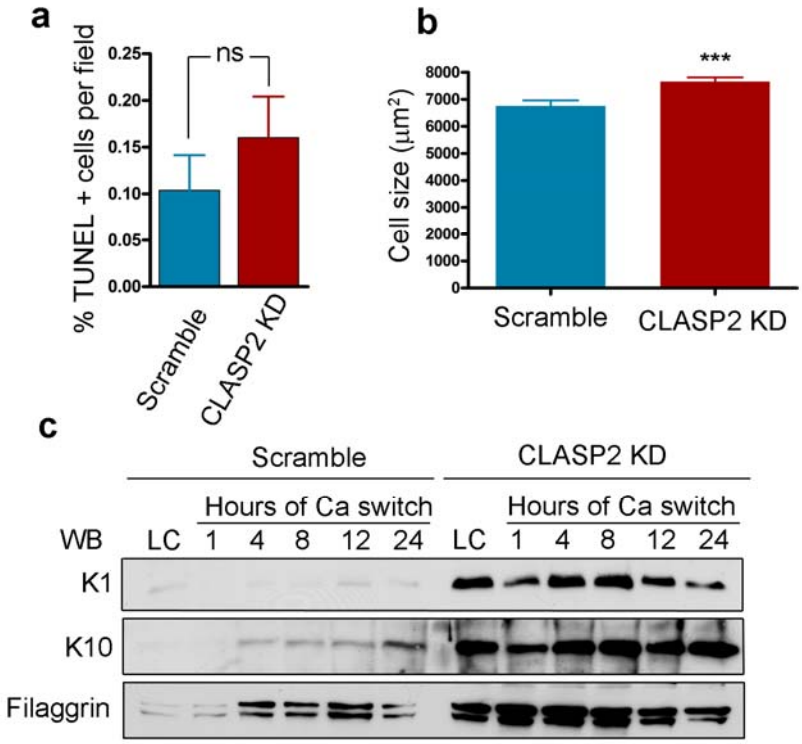


Figure 30 CLASP2-deficiency in mKer leads to an increased differentiation without an increase in apoptosis. (a) Scramble and CLASP2 KD mKer were assayed for the presence of apoptotic cells using TUNEL. The percentage of positive cells per field was quantified. Mann Whitney U test. ns: non-significant. **(b)** Scramble and CLASP2 KD mKer were stained with cell mask to mark all the cytoplasm and the size of the cells was quantified. Data is represented as mean ± s.e.m.; *** $p<0.0001$, Mann Whitney U test. **(c)** Immunoblot showing total levels of keratin 1 (K1), keratin 10 (K10) and filaggrin in Scramble and CLASP2 KD mKer both in low calcium conditions (LC) and at different time points of a calcium switch time course.

3.2. CLASP2-p120 interaction at non-adhesion sites

The cause underlying the observed alteration in the proliferation-differentiation balance in absence of CLASP2 could be related to the described alterations in AJs. However, since an absence of both ECad and PCad in the epidermis does not cause alterations in the proliferation/differentiation balance of mKer (Tinkle et al., 2008), other mechanisms may underlie this defect.

Interestingly, p120-null mKer present spindle alterations *in vitro* as well as a decrease in proliferation, yet the mechanisms are not completely understood (Perez-Moreno et al., 2008). Given the similarities with the CLASP2 deficient phenotype, we reasoned that the CLASP2-p120 interaction might occur at other potential locations in the cell to control the mKer proliferation/differentiation balance.

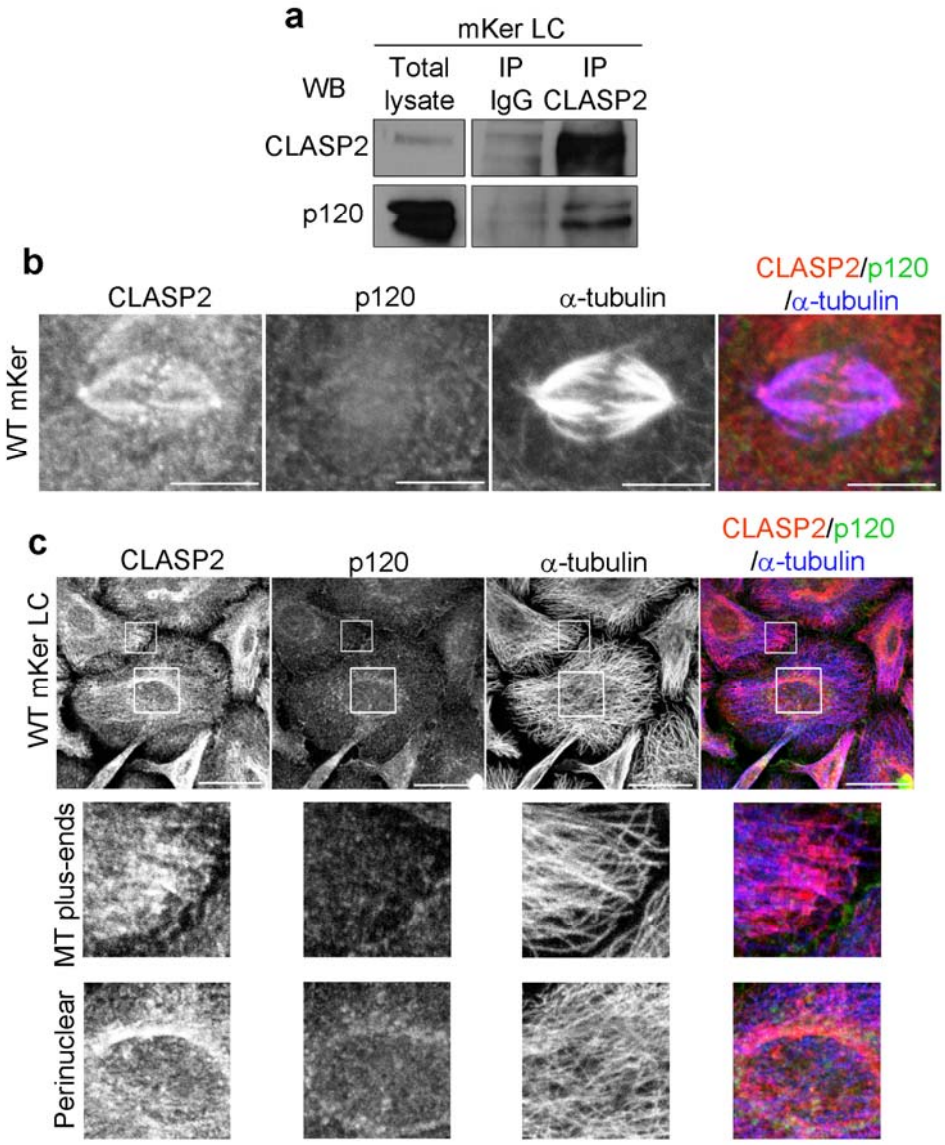


Figure 31 CLASP2 and p120 interact in absence of cell-cell contacts but do not colocalize at mitotic spindles or MT plus-ends. (a) CLASP2 was immunoprecipitated from mKer grown in low calcium conditions (LC). The immunoprecipitates were blotted for CLASP2 and p120. **(b)** WT mKer stained for CLASP2, p120 and α-tubulin. Scale bar, 7.5 μm. **(c)** WT mKer immunostained for CLASP2, p120 and α-tubulin. Scale bar, 25 μm. Insets corresponding to MT plus-ends and the perinuclear region are shown.

We have seen that catenins are promiscuous molecules in terms of functions and localizations (Perez-Moreno and Fuchs, 2006). We thus evaluated whether CLASP2 and p120 could interact at non-adhesion sites. CLASP2 and p120 were co-immunoprecipitated in mKer grown in low calcium (Fig. 31a) suggesting that they can interact in the absence of stable cell-cell contacts. In an attempt

to identify the localization of the potential interaction, we evaluated their distribution in mKer in low calcium conditions. However, we did not observe colocalization neither at MT plus-ends nor at mitotic spindles (Fig. 31b, c). A small degree of colocalization was observed at the perinuclear region (Fig. 31b).

It has been previously shown that cytoplasmic p120 localizes to centrosomes in tumor cell lines both during interphase (Chartier et al., 2007) and mitosis (Franz and Ridley, 2004) and this may be the result of an increase in the levels of cytoplasmic p120. Thus, we decided to investigate with more detail whether CLASP2 and p120 were found together at centrosomes. In order to have a clean system to study the CLASP2-p120 interaction in absence of cadherins we decided to use mouse L-cell fibroblasts since these cells do not express cadherins and do not form cell-cell contacts unless cadherins are exogenously expressed (Nagafuchi et al., 1987).

As done previously with mKer, we immunoprecipitated CLASP2 from a lysate of L cells. The immunoprecipitates contained not only p120 but also γ-tubulin (Fig. 32a)[2]. This result suggests that CLASP2 and p120 may also interact at MT nucleation sites, particularly the centrosome, since CLASP2 is a known centrosomal protein (Efimov et al., 2007; Lanza et al.; Pereira et al., 2006). Indeed we could identify CLASP2 colocalizing with γ-tubulin by immunofluorescence (Fig. 32b) in L cells; however, we were unable to detect endogenous levels of p120 at the centrosome by immunofluorescence (data not shown). For this reason, we decided to isolate centrosomes using a sucrose-gradient as a proof of principle that both CLASP2 and p120 localize to centrosomes.

To isolate centrosomes, mouse embryonic fibroblasts (MEFs) were initially treated with 10 μg/mL of nocodazole and 5 μg/mL of cytochalasine-D and subsequently lysed with a hypotonic buffer. The fractions collected from the sucrose gradient were analyzed for γ-tubulin as the main constituent of the centrosome and nuclear and cytoplasmic contamination was excluded with immunoblots for actin and N-Cadherin (NCad) (Fig. 32c). Fractions positive for actin and γ-tubulin were excluded since most likely they represent non-centrosomal accumulations of γ-tubulin. From the four centrosomal fractions detected, the last one was positive for both p120 and CLASP2 (Fig. 32c). This result indicates that p120 and CLASP2 localize at the centrosome in fibroblast cells, and can potentially interact at this location. Even though we have not detected endogenous p120 at the centrosome by immunofluorescence (Fig. 31b), our results are in agreement with previous publications showing p120 localization to the centrosome by isolation of centrosomes (Chartier et al., 2007). Whether the potential interaction takes place at the interphase centrosome or at the

[2] Note that from the two major isoforms expressed in L cells, only one seems to coimmunoprecipitate with CLASP2 and γ-tubulin

mitotic centrosome remains to be further explored. We further tried to isolate centrosomes from WT MEFs (p120 f/f) and p120-null MEFs (p120 Δ/Δ) to determine whether p120 is necessary for CLASP2 localization to the centrosome. In order to do so, p120 expression was ablated by infecting the cells with retroviruses expressing Cre-GFP following selection with puromycin. However, we were unable to effectively deplete p120 from the centrosomes (data not shown).

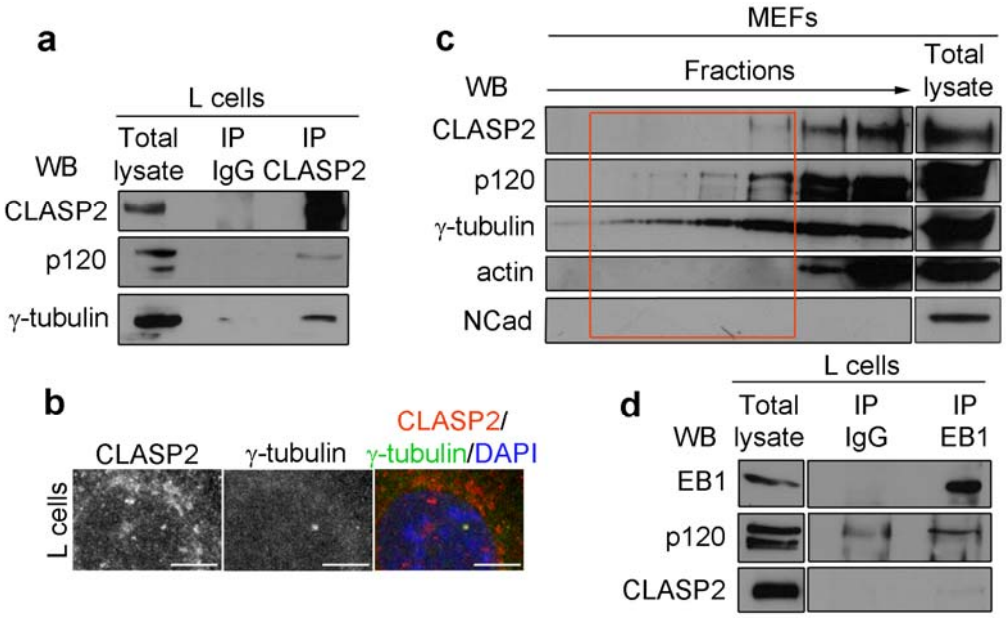


Figure 32 CLASP2 and p120 localize at the centrosome in fibroblasts. (a) CLASP2 was immunoprecipitated (IP) from a lysate of L cells. The IPs were analyzed by immunoblot for CLASP2, p120 and γ-tubulin. **(b)** L cells immunostained for CLASP2 and γ-tubulin. Scale bar, 5 μm. **(c)** Centrosomes were isolated from Mouse Embryonic Fibroblasts (MEFs) using a sucrose gradient. Representative fractions from the gradient are shown. NCadherin (NCad) and actin were used as controls for membrane and cytoplasmic contamination respectively. The centrosome-containing fractions are γ-tubulin positive and actin/NCad negative (red box). The presence of p120 and CLASP2 in the fractions was analyzed. **(d)** EB1 was immunoprecipitated from L cells following immunoblot for EB1, p120 and CLASP2.

Interestingly, when EB1 was immunoprecipitated from L cells both CLASP2 and p120 were recovered in the immunoprecipitates (Fig. 32d). EB1 is a key player for the function of other plus-end binding proteins (Morrison, 2007) and it is also a component of the mammalian centrosome (Louie et al., 2004), thus, these interactions could take place both at the centrosome, as mentioned before, or at MT plus-ends.

Next, we tried to extrapolate the results obtained in fibroblasts to our mKer. However, we were unable to coimmunoprecipitate CLASP2 and p120 with neither γ-tubulin nor EB1 (data not shown). We could neither isolate centrosomes since mKer seemed to be resistant to the hypotonic buffer

used to lyse the cells. Therefore, future studies will be needed to determine whether the CLASP2-p120 interaction can occur at MT plus-ends, the centrosome and/or the perinuclear region. Moreover, whether CLASP2 and p120 function co-coordinately at the level of mitotic spindles and centrosomes regulating cell division and proper spindle formation await further investigation.

The regulation of the centrosome could also provide a polarity cue that could explain the observed alteration in the proliferation-differentiation balance in absence of CLASP2 as a potential upstream requirement for spindle orientation. As discussed in the introduction, spindle orientation is one of the major determinants of cell fate in the epidermis, and alterations in spindle positioning lead to a proliferation-differentiation misbalance (Ray and Lechler, 2011). Some MT plus-end binding proteins are part of the machinery required to rotate the spindle (Gundersen et al., 2004) and, in addition, a role for cadherin-based adhesion in positioning of the mitotic spindle and polarization of the centrosome has been recently acknowledge (den Elzen et al., 2009; Desai et al., 2009; Dupin et al., 2009; Inaba et al., 2010). These observations raise the possibility that anchorage of astral MTs to cadherin-based adhesions via the p120-CLASP2 interaction controls spindle orientation in epidermal progenitor cells. To test this possibility we needed to investigate CLASP2 function in a more physiological scenario since cell fate and spindle orientation are regulated by a number of extrinsic factors coming from the tissue microenvironment (Ray and Lechler, 2011). In the next section we will describe CLASP2 function in the maintenance of epidermal homeostasis.

4. Study the role of CLASP2 in the maintenance of epidermal homeostasis

4.1. CLASP2 localizes to cortical sites in basal progenitor cells of the epidermis

4.1.1. CLASP2 expression is enriched in basal epidermal cells

We have demonstrated that CLASP2 is required to maintain AJs stability and the proliferation-differentiation balance of mKer *in vitro*. At the level of AJs it acts coordinately with p120, likely regulating MT targeting and AJs dynamics. We moved forward, and decided to extrapolate our *in vitro* mechanisms to an *in vivo* situation. Since CLASP2 controls the proliferation-differentiation balance *in vitro,* is it enriched in basal progenitor epidermal cells *in vivo*? Does it localize to AJs in basal epidermal cells?

By immunostaining CLASP2 in backskin from WT newborn mice we observed that it was enriched in basal progenitor cells of the interfollicular epidermis (Fig. 33a). In these cells it presented a cortical localization, in many cases overlapping with p120, but also extending in the cortical cytoplasm (Fig. 33a). Of note, we could also observe CLASP2 enrichment in basal progenitor cells of other stratified epithelial tissues such as the tongue (Fig. 33b), as well as in progenitor cells of simple epithelial tissues, such as in the very-well known stem cell compartment of the intestinal crypts (Fig. 33b). Supporting our findings, a role for CLASP2 in the maintenance of hematopoietic progenitor cells has been recently reported (Drabek et al., 2012).

To validate this result, we isolated by FACS progenitor cells from the epidermis. Briefly, mKer directly obtained from newborn mice were FACS-sorted according to their levels of α6-integrin, a very-well known marker of basal epidermal cells (Sonnenberg et al., 1991). RT-PCR analyses of $\alpha6^{high}$ and $\alpha6^{low}$ populations revealed that CLASP2 expression was two folds higher in basal mKer (Fig. 33c). Keratin 5 (K5) and loricrin were used as controls of basal and suprabasal mKer respectively. This decrease in CLASP2 expression induced upon differentiation was also observed *in vitro*. By immunoblot we could validate that long time treatment with calcium led to a significant decrease in the levels of the CLASP2 protein (Fig. 33d, e).

Globally, our results show that CLASP2 expression is enriched in progenitor epithelial cells, supporting our hypothesis that CLASP2 plays a role in maintaining epidermal homeostasis. This finding is particularly interesting given the MT organization of the epidermis (Simpson et al., 2011). As mentioned in the introduction, in basal progenitor cells of the epidermis MTs grow from the

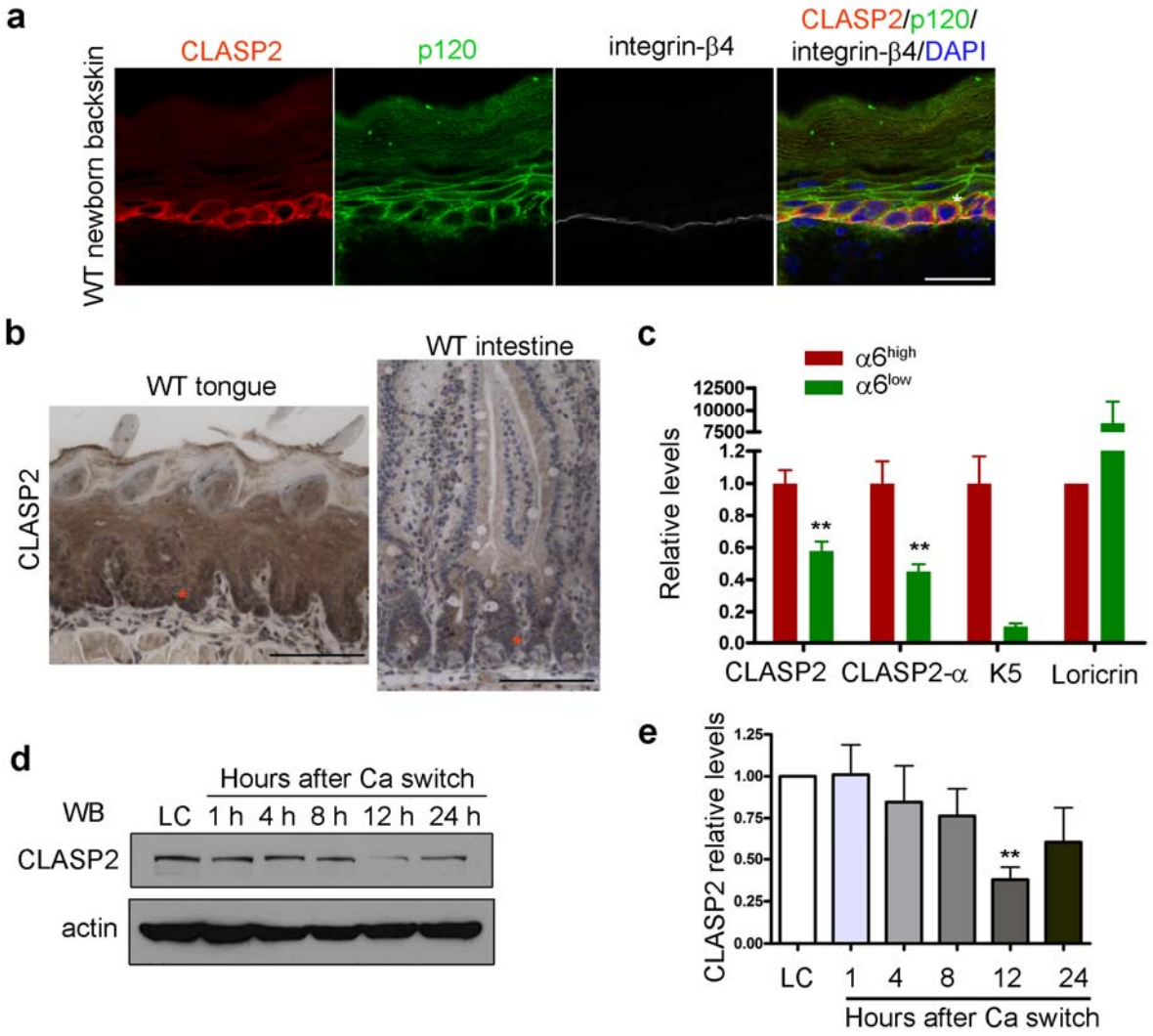


Figure 33 CLASP2 expression is enriched in progenitor basal epidermal cells. (a) WT newborn backskin immunostained for CLASP2, p120 and integrin-β4. epi: epidermis, derm: dermis. Scale bar, 25 μm. **(b)** WT adult tongue and WT adult intestine stained for CLASP2. Asterisks indicate CLASP2 enrichment in the basal layer of the tongue epithelium and the intestinal crypts, respectively. Scale bar, 100 μm. **(c)** Real time PCR analysis of mKer isolated from the backskin of newborn mice and FACS sorted according to their levels of α6-integrin into two populations: $\alpha6^{high}$ (basal mKer) and $\alpha6^{low}$ (suprabasal mKer). CLASP2 levels were analyzed with two different combinations of primers. Keratin 5 (K5) and loricrin were used as markers of basal and suprabasal keratinocytes respectively. For each primer pair, values were normalized to the transcript levels of the $\alpha6^{high}$ population. Data is represented as mean ± s.e.m.; * $p<0.01$, Student's t test. **(d)** Immunoblot analysis of CLASP2 levels in WT mKer subjected to a calcium switch time course for the time points indicated in hours. LC: low calcium. **(e)** Quantification of CLASP2 levels observed by immunoblot in panel d. n = 3 independent experiments. Data is represented as mean ± s.e.m.; * $p<0.004$, Student's t test.

centrosome projecting their plus ends towards cortical sites (Lechler and Fuchs, 2007). Thus, we propose that in this specific compartment of the epidermis, CLASP2-decorated MTs are targeted to AJs via the p120-CLASP2 interaction.

4.1.2. Nezha localizes to the cortex of suprabasal epidermal cells

If CLASP2 expression is decreased upon differentiation, how are MTs targeted to AJs in suprabasal compartments? In cell lines derived from simple epithelial tissues, it has been described that p120 is able to interact with MT minus-ends via the novel MT minus-end binding protein Nezha (Meng et al., 2008). Given the fact that MT minus-ends are not anchored at the centrosome in suprabasal keratinocytes (Lechler and Fuchs, 2007), we explored whether Nezha could be implicated in minus-end MT anchorage to AJs in suprabasal cells. Immunostaining of Nezha in epidermal sections from newborn mice showed that Nezha localized to cortical sites specifically in suprabasal epidermal cells (Fig. 34a), opposite to CLASP2 localization.

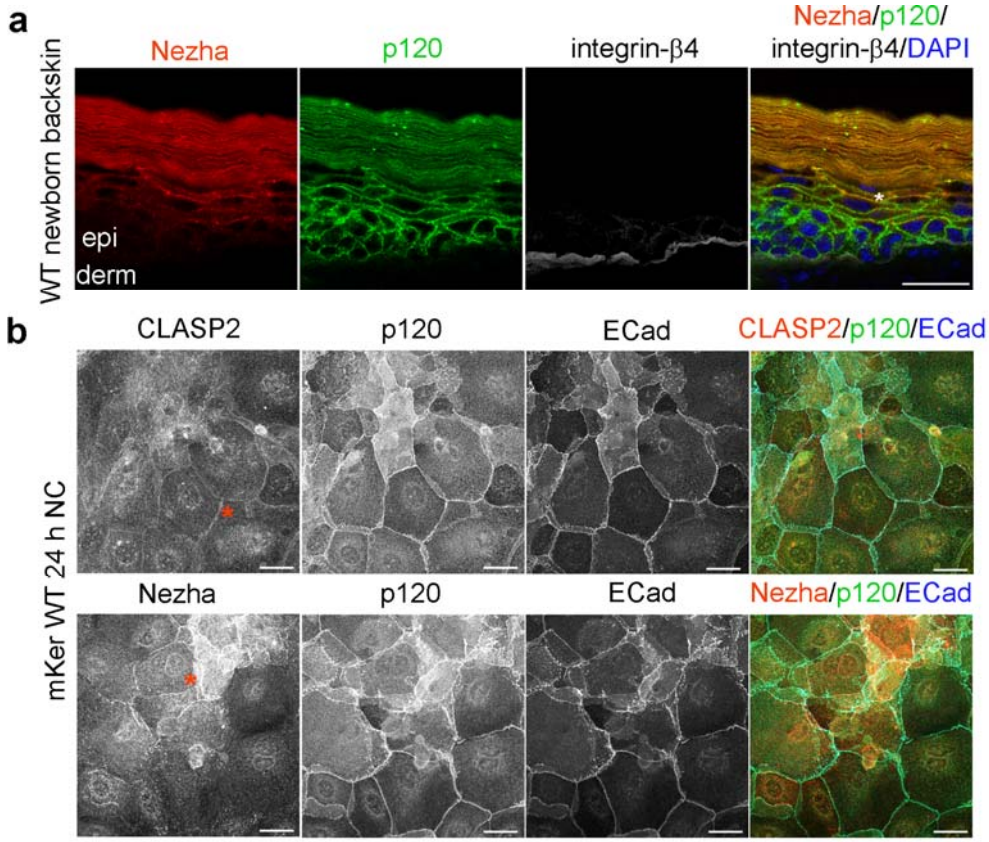


Figure 34 CLASP2 localizes to the cortex of basal progenitor epidermal cells whereas Nezha localizes to the cortex of suprabasal epidermal cells. (a) Backskins from WT newborn mice stained for Nezha, p120 and integrin-β4. The asterisk indicates suprabasal Nezha localization. Scale bar, 25 μm. **(b)** WT mKer subjected to a 24 hours calcium switch *in vitro* and immunostained for either CLASP2 or Nezha together with p120 and ECad. The asterisks point to a basal cell with CLASP2 at AJs and a differentiated cell with AJs-associated Nezha respectively. Scale bar, 25 μm.

We validated this result *in vitro*. After 24 hours of stimulation with calcium-containing media, mKer already express differentiation markers and cells with suprabasal characteristics can be identified in the culture. These cells showed clear membrane localization of Nezha, whereas more "basal" cells showed clear CLASP2 membrane staining (Fig. 34b). Unfortunately, since both CLASP2 and Nezha

antibodies were generated in rabbits, this limited the possibility to test whether the cortical localization of these proteins was mutually exclusive.

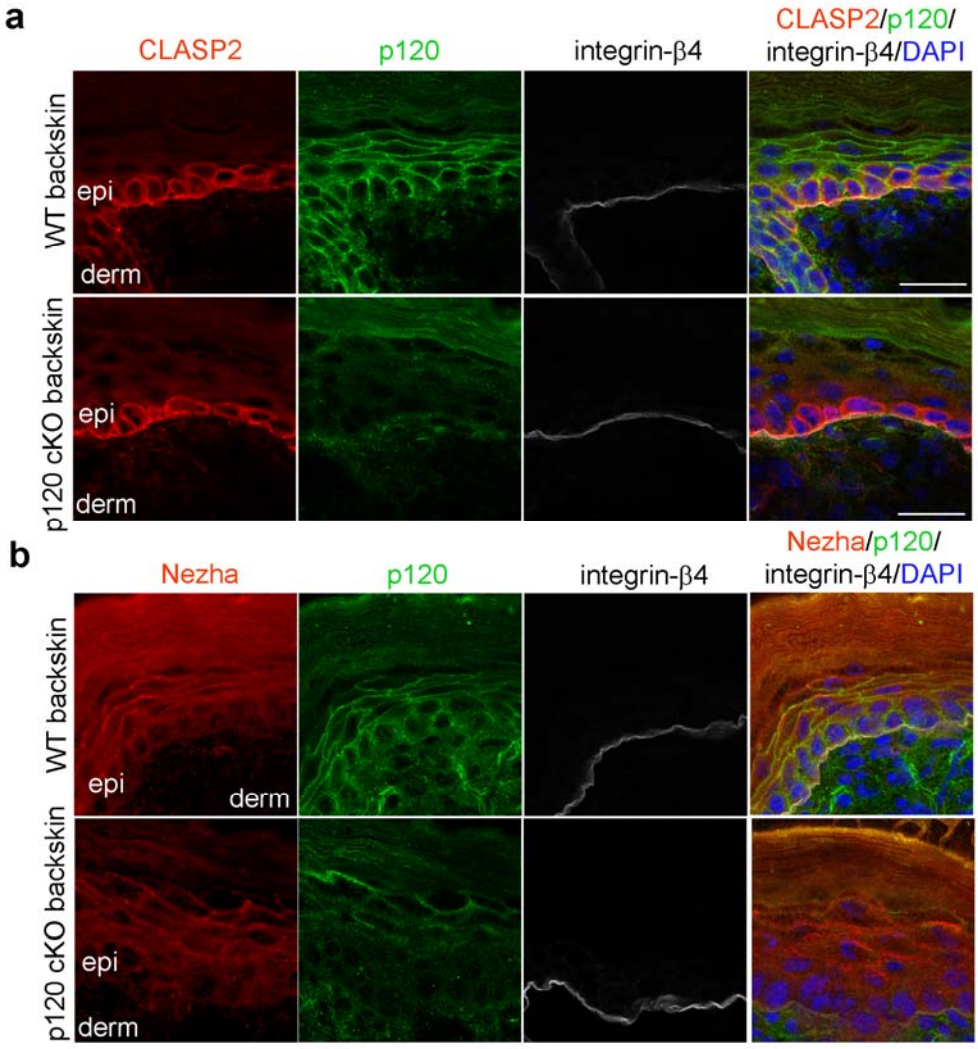


Figure 35 CLASP2 and Nezha localization to cell-cell contacts in epidermal cells is not affected in absence of p120. (a) Backskin sections from WT newborn mice and p120 conditional KO mice (cKO) stained for CLASP2, p120 and integrin-β4. Scale bar, 25 µm. epi: epidermis, derm: dermis. **(b)** Backskin sections from WT newborn mice and p120 conditional KO mice (cKO) stained for Nezha, p120 and integrin-β4. Scale bar, 25 µm. epi: epidermis, derm: dermis.

We therefore propose that CLASP2 targets MT plus-ends to AJs in basal epidermal progenitor cells via interaction with p120, whereas MT minus-ends are targeted to AJs in suprabasal cells via the Nezha-p120 interaction. However, we could not detect any clear alterations in the cortical localization pattern of either CLASP2 or Nezha in the skin from p120 conditional KO mice (Fig. 35 a, b). This could either suggest that other mechanisms exist for proper CLASP2/Nezha targeting to AJs,

as mentioned before (Fig. 12); or, alternatively, that members of the p120 family of proteins such as ARVCF compensate in the epidermis for the loss of p120 (Perez-Moreno et al., 2006).

4.1.3. CLASP2 expression is increased during epidermal development

Epidermal basal progenitor cells are responsible for giving rise to the differentiated layers of the epidermis during epidermal development and for maintaining proper epidermal homeostasis in adult tissues (Blanpain and Fuchs, 2009). We have seen that CLASP2 expression is enriched in basal progenitor cells of the epidermis. At this location it may fulfill two functions: maintenance of proper AJ dynamics through MT targeting to cell-cell adhesion sites, and positioning of the mitotic spindle during cell division; thus, controlling the balance between proliferation and differentiation of progenitor epidermal cells. In this regard, CLASP2 expression, analyzed by RT-PCR, was increased during epidermal development (Fig. 36a). At the time points analyzed extensive proliferation of the epidermis takes places to generate the differentiated layers of the epidermis, possibly by asymmetric cell division (Lechler and Fuchs, 2005; Williams et al., 2011). Equivalent results were observed by immunostaining CLASP2 at different time points of epidermal development (Fig. 36b). At embryonic day 14.5 (E14.5) CLASP2 presented an apical localization within basal cells of the epidermis, most likely corresponding to the centrosome (Lechler and Fuchs, 2007). At E15.5 CLASP2 was clearly enriched in basal progenitor cells, and it remained enriched in this compartment throughout epidermal development, although its levels slightly decreased (Fig. 36a, b).

4.2. Generation of a CLASP2 conditional KO mouse model

The results described until now indicate that CLASP2-deficiency *in vitro* leads to defects in AJs dynamics and alters the balance between proliferation and differentiation. In addition, we have made the interesting observation that CLASP2 is enriched in epidermal progenitor cells. Therefore, we hypothesize that CLASP2 may control proper AJs dynamics in progenitor epidermal cells. Furthermore, by interacting with AJs components, it can potentially have an impact on spindle orientation (den Elzen et al., 2009; Inaba et al., 2010) and thus, on the balance between proliferation and differentiation of progenitor epidermal cells.

To test our hypothesis, we decided to generate a CLASP2 conditional KO mouse model in the epidermis. As mentioned in the introduction, a full CLASP2 KO mouse had already been generated (Drabek et al., 2006) using gene-trap technology at the time we began our project. In this mouse model the second exon common to all three CLASP2 isoforms (exon 9) was chosen for generation of

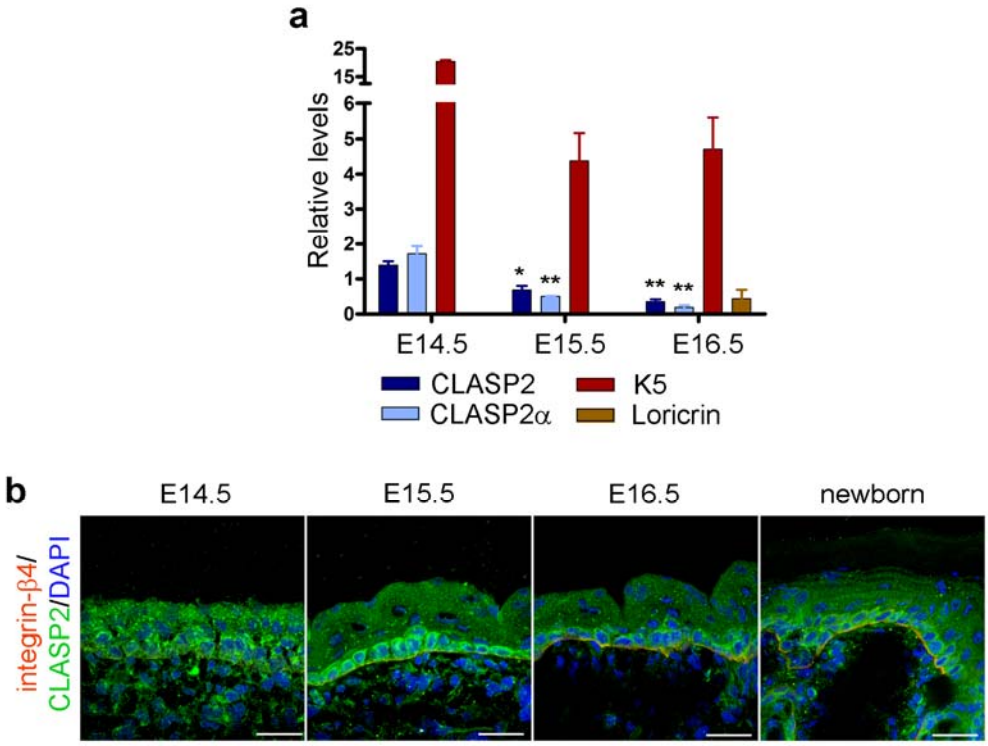


Figure 36 CLASP2 expression is increased at early time points of epidermal development. (a) RT-PCR analysis showing the levels of CLASP2, CLASP2α, Keratin 5 (K5) and loricrin in the epidermis of embryos at different time points of development. Data is represented as mean ± s.e.m.; * p<0.05, ** p<0.006, Student's t test. **(b)** Sections from embryos at different time points of development immunostained for integrin-β4 and CLASP2. Scale bar, 25 μm. E: embryonic day.

the targeting construct (Drabek et al., 2006). Since the previous exon (exon 8) is separated by a small intron (375bp) we decided to target not only the first common exon to all three isoforms but also the previous exon, specific of the short β and γ isoforms (Fig. 37a). After electroporation of ES cell clones we obtained 28 positive clones by Southern Blot (SB) for the 5' arm (Fig. 37b). From these 28 clones, we were able to confirm the occurrence of homology recombination with the 3' arm SB as well as with an internal control SB for the neo cassette in 2 clones -clone26 and clone142- (Fig. 37b). Genotyping for the 5' LoxP site gave equivalent results (Fig. 37c). Additionally we verified that infection of ES cell clones with lenti-Cre-GFP led to the deletion of the gene as observed by PCR (Fig. 37d).

Clones 26 and 142 were selected for aggregation, but only the clone 142 underwent germ-line transmission. A founder mouse was identified by Southern Blot and genotyping (data not shown) and the neo cassette was removed by crossing our mice with a flippase (FLP)-expressing mouse. Once homozygous flox/flox animals were obtained, they were crossed to mice expressing the Cre

recombinase under the K5 promoter, which specifically ablates the gene in epithelial tissues (Ramirez et al., 1994). The deletion of the gene was validated by PCR: the Δ band was specific for animals harboring the K5-Cre allele (Fig. 38a).

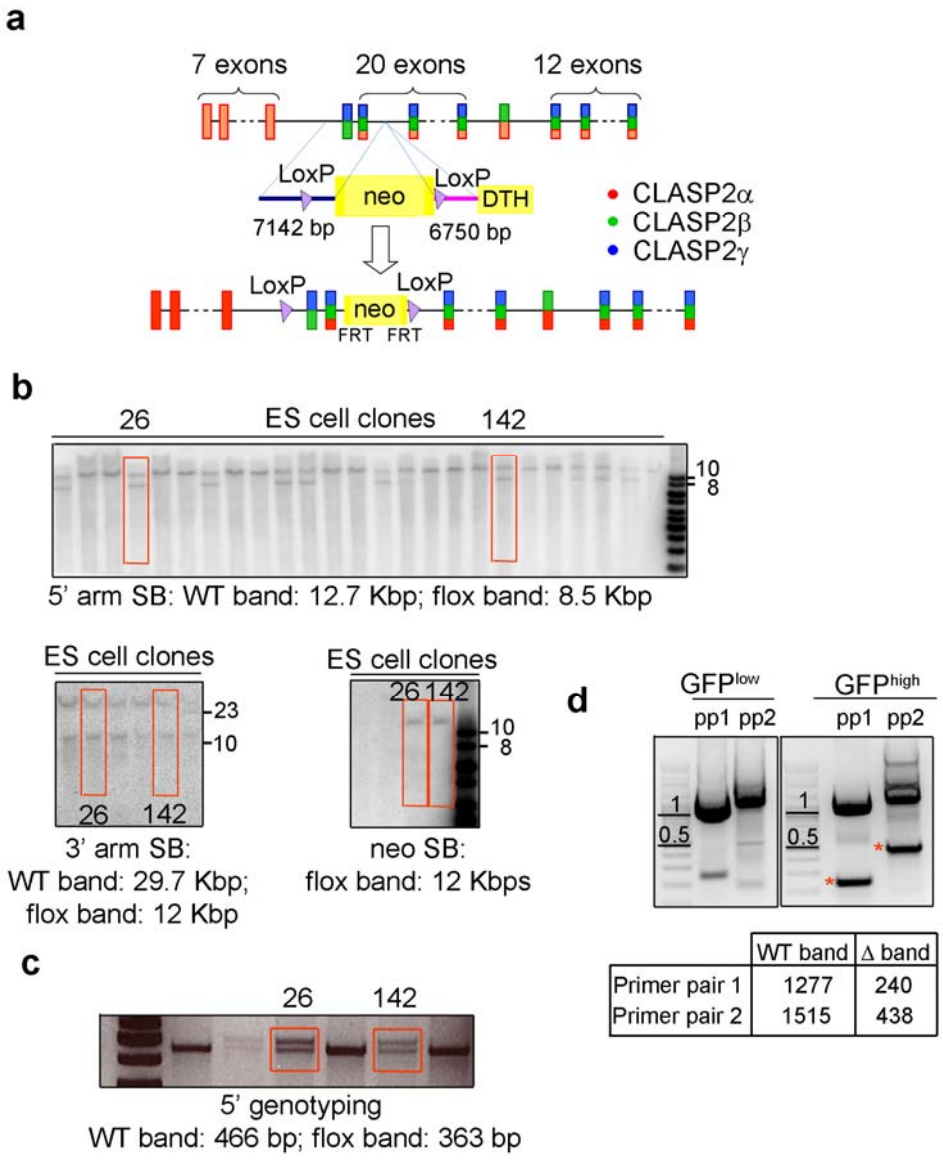


Figure 37 Generation of a CLASP2 conditional KO mouse. (a) Strategy for the generation of the targeting construct. The size of the homology arms is indicated. **(b)** Representative 5' arm, 3' arm and neo Southern Blots (SB) for some of the ES cell clones after electroporation of the targeting construct. Clones 26 and 142 were chosen for subsequent aggregation. **(c)** 5' genotyping of selected ES cell clones. (d) ES cell clones 26 and 142 were infected with adeno-Cre-GFP in vitro and FACS-sorted into two populations: GFP^{high} and GFP^{low}. The deletion of the gene was evaluated by PCR. pp: primer pair. Red boxes indicate the two clones selected for agreggation: clone 26 and clone 142. Expected molecular sizes are indicated.

Animals were born at expected Mendelian ratios and did not present any obvious macroscopic phenotypes (data not shown). Keratinocytes from the tail were isolated to validate the deletion of

the gene immunoblotting. However, CLASP2 expression levels did not change in genetically KO mice (CLASP2$^{\Delta ep}$)[3] (Fig. 38b). Analyzing the immunoblots carefully, we observed that in CLASP2$^{\Delta ep}$ mice the band corresponding to CLASP2 migrated slightly faster than the WT CLASP2 (Fig. 38b). This raised the possibility that deletion of the two targeted exons may have induced an aberrant protein by alternative splicing, that lacks the sequence corresponding to the two deleted exons (109 aa)[4]. In this regard, the CLASP2 mRNA sequence generated upon deletion of the two targeted exons, maintained the CLASP2 coding frame (data not shown). Thus, deletion of the two targeted exons generated a CLASP2 Knock-In (KI) mouse instead of a loss-of-function KO mouse.

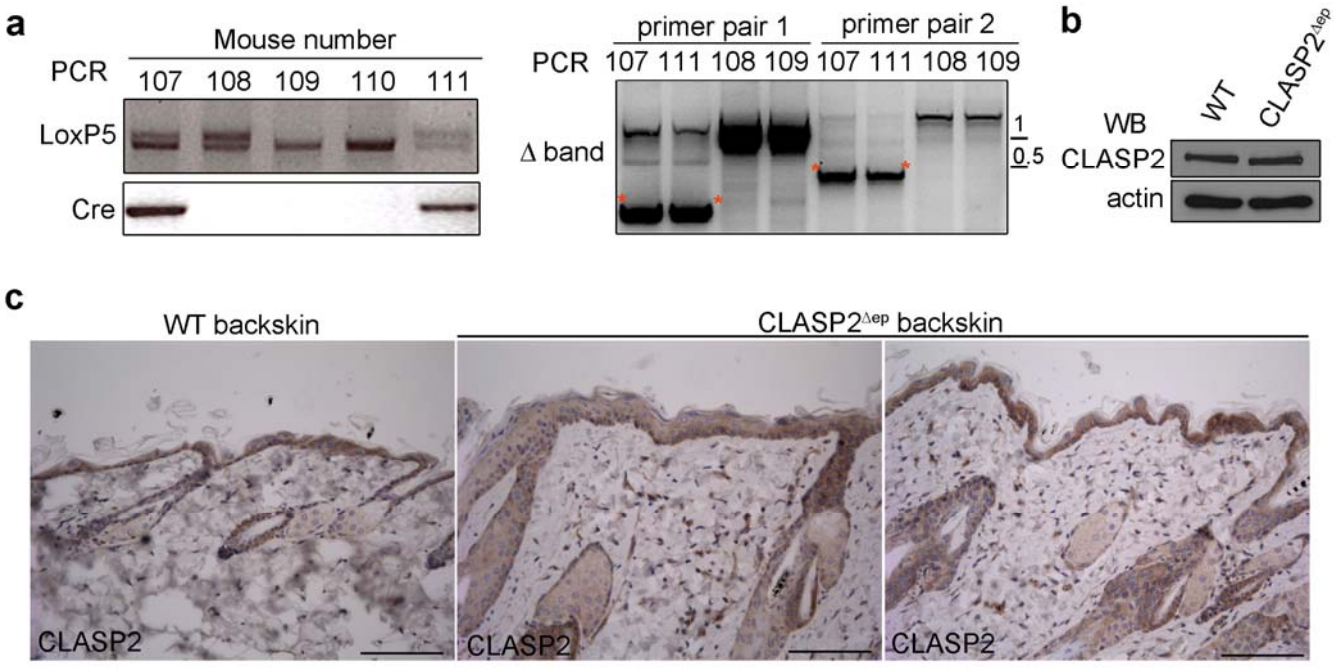


Figure 38 Genetic deletion of exons 8 and 9 using the Cre-LoxP system does not lead to loss of CLASP2 expression. (a) Genotyping PCR showing the presence of heterozygous lox/+ mice harboring the K5-Cre recombinase allele. These animals showed a Δ band reflecting deletion of exons 8 and 9 (asterisk). Each number represents a different mouse. **(b)** Protein lysates prepared from mKer isolated from tails of adult mice were immunoblotted for CLASP2. The genotype of the mice is the following: WT mice: CLASP2 flox/flox, K5-Cre negative; CLASP2$^{\Delta ep}$ mice: CLASP2 flox/flox, K5-Cre positive. n = 2 mice per genotype. (c) CLASP2 immunohistochemistry performed in skin samples from WT and CLASP2$^{\Delta ep}$ mice. Scale bar, 100 µm. Note the nomenclature "CLASP2$^{\Delta ep}$" refers exclusively to a genetic deletion. n = 2 mice per genotype

[3] Note that throughout the text the nomenclature CLASP2$^{\Delta ep}$ refers to a genetic deletion of CLASP2 that does not correspond to a loss of expression of the protein. Instead, the genetic manipulation generated a CLASP2 KI mouse.

[4] Note that the antibody recognizes the C-terminal region of the protein, thus, it could also recognize a CLASP2 variant lacking the two targeted exons.

We also analyzed the presence of the protein in the epidermis by immunohistochemistry, and the expression of CLASP2 in genetically CLASP2$^{\Delta ep}$ mice was still detected (Fig. 38c). Surprisingly, mice expressing the CLASP2 mutant protein showed a clear phenotype, with patches of increased epidermal thickening. This result suggests that the 109aa removed, corresponding to exons 8 and 9, may play a potential role for the correct functionality of the protein. The validation of this hypothesis will be explored in future studies since at present it is beyond the scope of this thesis project.

4.3. CLASP2 expression in skin carcinogenesis

Unfortunately we were not able to generate a loss-of-function mouse model to study the functional relevance of CLASP2 *in vivo*. However, given that *in vitro* we had seen alterations in proliferation and differentiation in absence of CLASP2 and its expression appeared to be increased in epidermal progenitor cells, we analyzed whether changes in its expression levels correlated with skin tumorigenesis.

Initially we used a well-characterized panel of mKer cell lines with increasing levels of malignancy, ranging from immortalized non-tumorigenic to highly malignant (Navarro et al., 1991). We could confirm that the most malignant cell lines showed a downregulation of ECad expression (Fig. 39a) (Navarro et al., 1991) as well as a slight decrease in p120 levels (Fig. 39a). Interestingly, CLASP2 expression progressively decreased from the less tumorigenic cell lines (MCA3D) to the most malignant ones (CarC) (Fig. 39a).

Additionally, we analyzed CLASP2 expression in squamous cell carcinomas (SCCs) from mice with different genetic backgrounds. As mentioned in the introduction, p120 deficiency correlates with human skin tumorigenesis (Perez-Moreno et al., 2008). Furthermore, p120 plays a causal role in mouse tumorigenesis, since engraftment of skin from p120$^{\Delta ep}$ animals into immunocompromised mice leads to development of SCCs (Perez-Moreno et al., 2008). We analyzed CLASP2 expression in SCCs derived from the skin of p120$^{\Delta ep}$ mice, and, corroborating the results obtained with the cell lines *in vitro*, we observed a significant decrease in the expression levels of CLASP2 (Fig. 39b).

We also analyzed CLASP2 levels in samples of SCCs obtained upon c-Fos overexpression in the epidermis and treatment with DMBA/TPA, a two-step chemically-induced skin carcinogenesis protocol (Filler et al., 2007), which were kindly provided by E. Briso de Montiano, CNIO, Madrid (Fig. 39c). DMBA/TPA treatment leads to the formation of skin papillomas, which represent benign epithelial tumors. Upon long DMBA/TPA treatment, these tumors may progress into malignant

SCCs. When the transcription factor c-Fos (member of the AP1 family of transcription factors) is expressed in the epidermis, the formation of SCCs upon DMBA/TPA treatment is accelerated (E. Briso de Montiano, unpublished data). CLASP2 was still expressed in papillomas from WT mice developed after DMBA/TPA treatment. However, its expression was significantly decreased in SCCs from c-Fos Tg mice treated with DMBA/TPA (Fig. 39c).

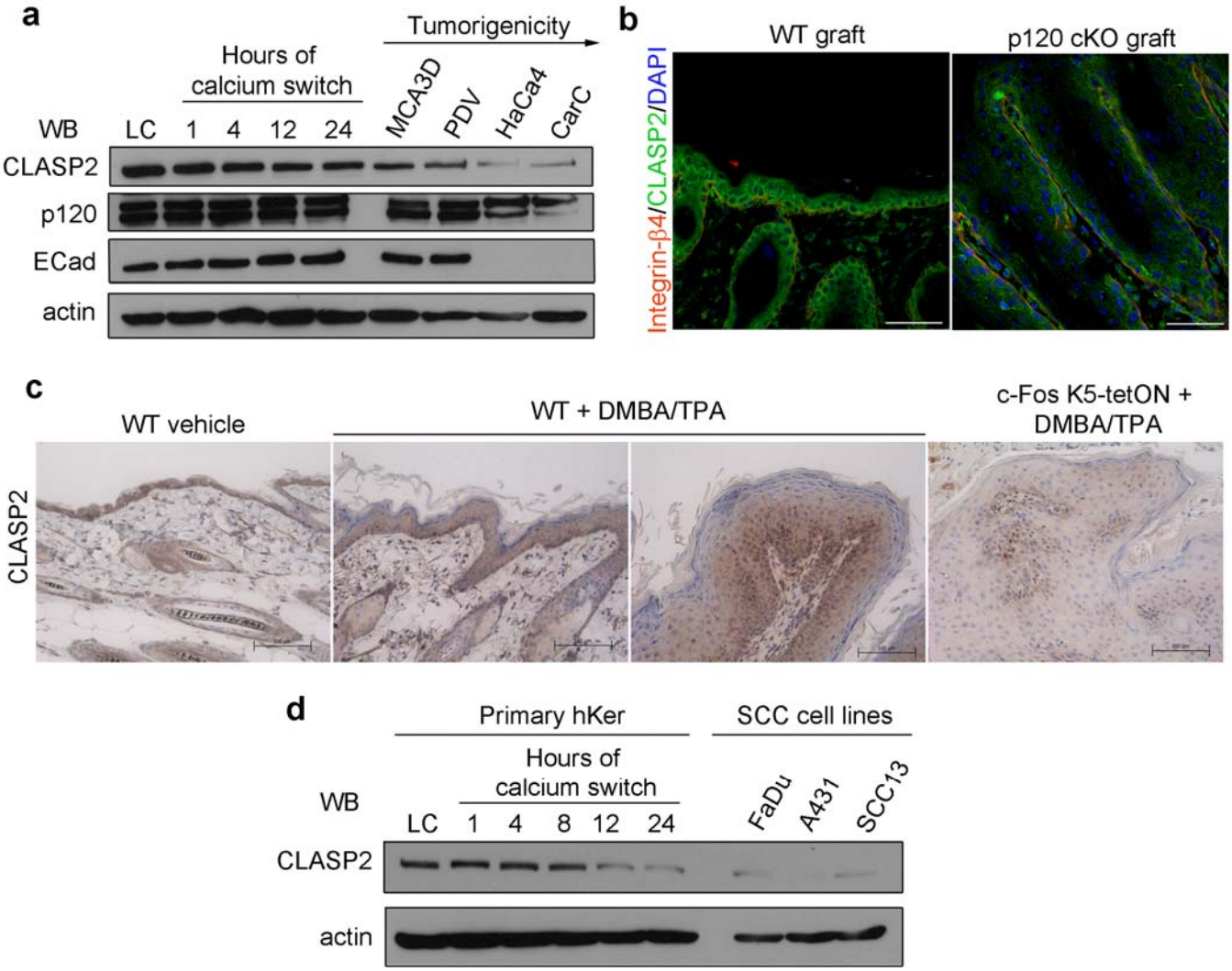


Figure 39 CLASP2 expression is downregulated in mouse SCCs and human SCC cell lines. (a) Immunoblot showing the levels of CLASP2, p120 and ECad in primary mKer in low calcium conditions (LC) and during a calcium switch time course experiment, as well as in a panel of keratinocyte cell lines with increasing tumorigenic characteristics. **(b)** Backskins from newborn WT or p120 cKO animals were grafted into immunocompromised mice and stained for CLASP2 and integrin-β4. Scale bar, 50 μm. **(c)** Skin sections from DMBA/TPA-treated c-Fos transgenic (K5-tetON) mice and WT littermates stained for CLASP2. Scale bar, 100 μm. **(d)** Analysis of CLASP2 levels in primary human keratinocytes (hKer) grown in LC or switched to calcium for specific time points, and three human squamous cell carcinoma lines (SCC cell lines).

We also analyzed CLASP2 expression in a panel of human SCCs cell lines obtaining equivalent results: CLASP2 expression was downregulated when compared to primary human keratinocytes (hKer) (Fig. 39d). Thus, we can conclude that CLASP2 expression is downregulated during mouse and human skin carcinogenesis.

Discussion

1. A model for the p120-CLASP2 interaction at Adherens Junctions

In this thesis we identified and characterized a novel interaction between the MT plus-end binding protein CLASP2 and the AJs component p120 in epidermal progenitor cells. This interaction is supported by loss of function studies, showing that absence of p120 leads to an altered localization of CLASP2 to sites of cell-cell adhesion. On the other hand, CLASP2 deficiency leads to alterations in p120 dynamics and, in broader terms, in AJs formation and functionality. Absence of either CLASP2 or p120 leads to MT alterations at the level of AJs, which suggests that their interaction could be responsible for targeting MTs to sites of cell-cell adhesion. The results obtained in this thesis project deepen our knowledge on the mechanisms mediating MT interactions with AJs in progenitor cells of the epidermis.

1.1. Microtubule targeting to Adherens Junctions via the p120-CLASP2 interaction

1.1.1. p120 as a regulator of microtubule targeting to Adherens Junctions

In a yeast-two hybrid screen we found a potential interaction between the AJs component p120 and the MT plus-end binding protein CLASP2. This raised the possibility that both proteins may function together at AJs regulating MT dynamics.

p120 is best known for its role as a regulator of cadherin stability at the membrane, but in the last decade a number of publications have reported an unexpected role for p120 in MT regulation. p120 has been shown to bind directly MTs and kinesin motors (Chen et al., 2003; Franz and Ridley, 2004; Yanagisawa et al., 2004), and to regulate MT dynamics in a cadherin-deficient neuroblastoma cell line (Ichii and Takeichi, 2007). Moreover, when bound to cadherins, p120 interacts with MT minus-ends via a complex formed by PLEKHA7 and the novel MT minus-end binding protein Nezha, leading to anchorage of MTs at AJs (Meng et al., 2008). Thus, p120 seems to be at the crossroads between adhesion and MTs.

1.1.2. CLASP2 as a cortical microtubule stabilizer

CLASP2 is an ideal candidate to mediate interactions between MT plus-ends and AJs. First, CLASPs (both CLASP1 and CLASP2) have been proposed to stabilize MTs at cortical sites in HeLa cells (Mimori-Kiyosue et al., 2005). According to the “search and capture” hypothesis, MT attachment to cortical sites leads to MT stabilization (Kirschner and Mitchison, 1986), and this has been characterized with detail in the leading edge of migrating polarized cells (Gundersen, 2002). Thus, we envisioned a working model in which MT targeting to AJs via the CLASP2-p120 interaction would

lead to selective MT stabilization at sites of cell-cell adhesion. Indeed, CLASPs have already been proposed to mediate cortical capture of MTs at cell-ECM adhesion sites, in the vicinity of FAs. CLASPs interact with the PIP3 binding proteins LL5α and LL5β, which form a complex around FAs in an integrin-activation dependent manner (Lansbergen et al., 2006). Absence of any of these proteins (integrins α3β1/α6β4, LL5s or CLASPs) leads to a decrease in MT density at cell-ECM adhesion sites and an increase in MT growth rate measured in terms of EB1-GFP dynamics (Hotta et al., 2010). The authors of this work proposed a model by which CLASPs-decorated MTs are targeted to cell-ECM adhesion sites via their interactions with LL5s. Our results expand the role of CLASP2 as a cortical MT anchor to AJs. Equivalent to the observed MT defects at cell-ECM adhesion sites, we observed a decrease in MT density and an increase in MT growth rate at AJs in absence of either CLASP2 or p120 (Fig. 24, 26).

The decreased MT density at AJs observed in absence of p120 seems to be specific of areas of cell-cell contact (Fig. 24), since we did not detect any significant differences in the number of cortical MTs in absence of calcium (data not shown). This finding is not consistent with previous results obtained by the group of A. Yap showing that expression of a mutated version of ECad unable to bind p120 did not lead to any alterations in MT density at adhesion sites (Stehbens et al., 2006). This difference may be due to cell type variability, since our experiments were done in primary cells and their experiments were conducted in cadherin-deficient Chinese hamster ovary cells (CHO cells) exogenously expressing ECad. We propose that in absence of p120, the observed MT alterations at AJs are a consequence of altered CLASP2 recruitment to the junctions.

It is important to mention that a number of connections between p120 and MTs have been previously documented. The interaction between p120 and the kinesin motor KIF5 (Yanagisawa et al., 2004) is unlikely to participate in the regulation of MT targeting to AJs. To our knowledge, there have not been reports of possible roles for KIF5 as a MT regulator, and most likely the p120-KIF5 interaction plays a role at the level of vesicle traffic. It has also been proposed that p120 interacts directly with MTs, and this interaction is mutually exclusive with the p120-cadherin interaction. Moreover, p120 seems to interact with MTs throughout the entire length of the MT polymer (Yanagisawa et al., 2004) leading to an increase MT stability in a cadherin-independent manner (Ichii and Takeichi, 2007). Thus, it is difficult to envision a model in which the direct interaction between p120 and MTs plays a role in MT targeting to adhesion sites. Additionally, p120 interacts with MT minus-ends via Nezha (Meng et al., 2008), and this could be another reason for the observed decrease in MT targeting to AJs. However, since basal mKer show a centrosomal MT organization (Lechler and Fuchs, 2007) these results do not favor this hypothesis.

Regarding CLASP2, the decreased MT density was observed both at areas of cell-cell contact as well as at cell free edges (Fig. 24 and data not shown). This is consistent with the decreased MT density

and MT stability observed in CLASP2 KO MEFs (Drabek et al., 2006). Rescue experiments are needed to verify whether the MT alterations observed at AJs in absence of either p120 or CLASP2 are a consequence of a loss of the interaction between both proteins, or just a general feature of CLASP2-deficient mKer. Importantly, in both cases, the observed alterations in MTs could also be a consequence of defects either in AJs stability or in the actin cytoskeleton (Fig. 24, 26, 27).

It has been proposed that MTs that reach areas of cell-cell contact are stabilized by interactions with cortical proteins, and this is in turn important for the stability of the junction (Komarova et al., 2012; Waterman-Storer et al., 2000). We envision a model of MT targeting and stabilization at cell-cell contacts mediated by the CLASP2-p120 interaction. Supporting this hypothesis, the number of nocodazole resistant MTs at AJs significantly decreased in absence of either CLASP2 or p120 (Fig. 25). It remains to be addressed whether this decrease in MT stability is specific of cell-cell contact areas or a general feature of CLASP2-deficient and p120 KO mKer. Interestingly, we did not observe any change in the levels of acetylated-tubulin and detyrosinated-tubulin was not detected (Fig. 25). Knock-down of CLASP2 by siRNA in HeLa cells does not lead to changes in the levels of acetylated-tubulin (Mimori-Kiyosue et al., 2005), whereas CLASP2 KO MEFs present a decrease in acetylated-tubulin (Drabek et al., 2006). Thus, it may be possible that a complete loss of the protein is required to observe a decrease in the total levels of acetylated-tubulin. Independently of the role of CLASP2 in determining the levels of acetylated-tubulin, it is clear from our experiments that formation of cell-cell contacts does not trigger an increase in acetylated-tubulin (Fig. 25). Regarding the formation of detyrosinated tubulin, it may be that mKer do not have the enzymes required for the generation of this modification, or alternatively, that detyrosinated-tubulin is specific of other MT organizations such as the one observed in suprabasal differentiated mKer.

1.2. Biochemical analysis of the p120-CLASP2 interaction

We have demonstrated that CLASP2 and p120 directly interact via the N-terminal regulatory domain of p120 and the N1 region of CLASP2 (Fig. 14, 15, 17). Interestingly, the N-terminal domain of p120 is subjected to multiple phosphorylation events (Alema and Salvatore, 2007), raising the possibility that Tyr or Ser/Thr phosphorylation of p120 regulates its association with CLASP2. Additionally, the p120 N-terminal domain has been implicated in KIF5 and PLEKHA7 binding. Thus, interaction of p120 with KIF5, PLEKHA7 and CLASP2 seems to be a conserved function of p120 isoforms 1 to 3. How is the association to such different MT related proteins regulated in time and space requires further investigation and will shed light on the differential effects of different types of MT binding proteins on AJs homeostasis.

Since all three proteins KIF5, PLEKHA7 and CLASP2 bind the N-terminal domain of p120, they can be part of a tripartite complex together with cadherins and p120. Indeed, we have seen that CLASP2,

p120 and ECad coimmunoprecipitate, both from protein total lysates and from lysates of purified surface proteins (Fig. 7).

Regarding CLASP2, we have identified the N1 region as responsible for binding p120. This region comprises both the Arg-Ser rich region as well as a TOG-like cryptic domain. A construct lacking the Arg-Ser rich region is able to weakly interact with p120 (Fig. 17). Thus, the region containing the TOG-like domain may be sufficient to bind p120, although the Ser-Arg rich domain likely reinforces the CLASP2-p120 interaction. Given that the Ser-Arg rich region is implicated in MT binding, CLASP2 may be able to bind both p120 and MTs. Indeed, co-staining of ECad, CLASP2 and α-tubulin at early time points of a calcium switch revealed that CLASP2-decorated MTs were targeted to AJs (Fig. 9a). In addition, to visualize individual MTs, we treated cells with 5 µM nocodazole. These resistant MTs were decorated by comets of CLASP2 in close contact with ECad positive sites (Fig. 9b). Globally, these experiments suggest that CLASP2 can simultaneously interact with p120 and MTs. However, a definitive proof of such a hypothesis would require pull-down assays in the presence of *in vitro* polymerized MTs.

1.3. Junctional CLASP2 localization

A major question was the identification of the mechanism by which CLASP2 is targeted to areas of cell-cell contact. From experiments using ECad-PCad deficient cells we learned that contact formation was required for the formation of a cortical CLASP2 enrichment (Fig. 6d). However, since formation of AJs is required for the proper assembly of other adhesion complexes such as desmosomes and TJs (Tinkle et al., 2008), it was important to show a role for cadherin-mediated ligation in the recruitment of CLASP2. To rule out the involvement of other adhesion complexes, we induced cell attachment to ECad-coated plates. Using this methodology we observed that cadherin ligation was sufficient to recruit CLASP2 to sites of cell-cell adhesion in absence of other type of signal (Fig. 6e). Specifically, p120 played a major role in the recruitment of CLASP2 to cell-cell contacts (Fig. 11, 18). However, it should be emphasized that although ECad overexpression in p120-deficient cells was not sufficient to fully rescue CLASP2 localization at AJs, we could observe a partial rescue (Fig. 12c, d). In agreement with such a partial rescue, treatment of p120-deficient cells with calcium for a long period to induce more robust AJs led to partial recruitment of CLASP2 to AJs (Fig. 12a, b). These experiments suggest that, besides p120, CLASP2 may associate with a series of AJs components which include: cortical actin (Tsvetkov et al., 2007), ACF7 (Drabek et al., 2006; Karakesisoglou et al., 2000; Lowery et al., 2010; Zaoui et al., 2010), IQGAP1 (Watanabe et al., 2009), or any other factor not yet characterized (Fig. 40). We will discuss in more detail the importance of these potential interactions in the following sections.

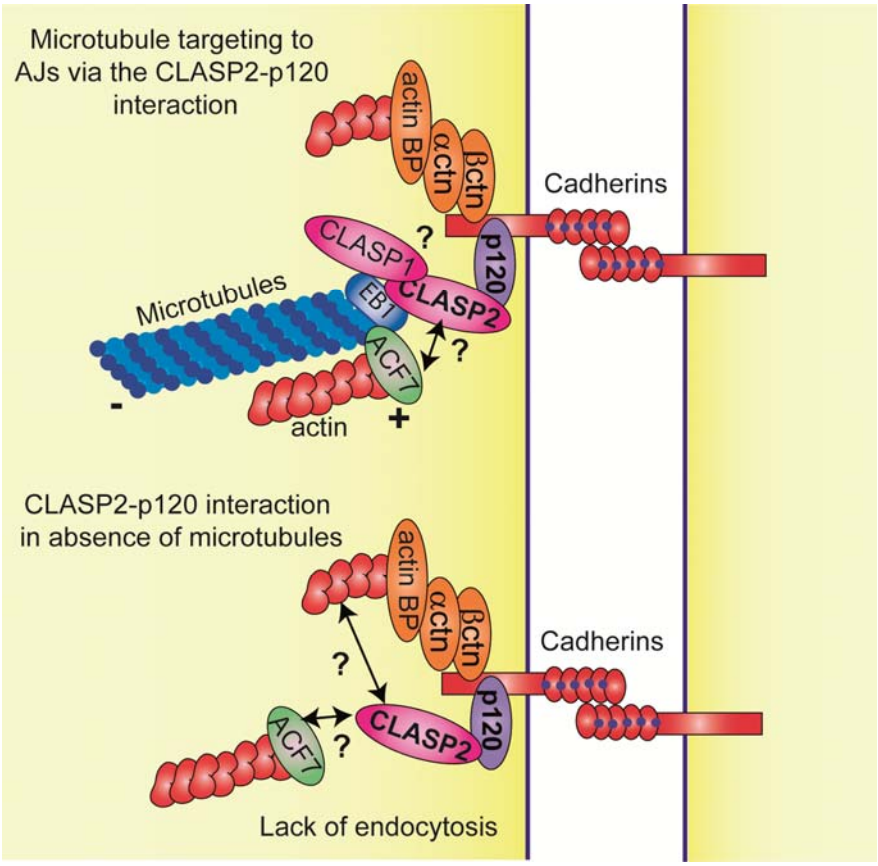


Figure 40 CLASP2 interactions with AJs components. CLASP2-decorated MTs are targeted to AJs via the interaction between CLASP2 and p120. This interaction controls AJs stability and MT behavior at sites of cell-cell adhesion. CLASP1 may associate with CLASP2 at these sites, yet whether it interacts with any other component of AJs remains to be explored. In absence of MTs, CLASP2 remains at AJs associated to p120. Whether CLASP2 also interacts with other AJs components such as cortical actin or ACF7 at cell-cell contacts remains to be evaluated. In the absence of MTs cadherins are not internalized and the AJs complex remains at the membrane.

2. Who needs microtubules?

2.1. Formation of Adherens Junctions

One of the most unexpected results obtained is the apparently normal assembly of AJs upon calcium switch in cells lacking MTs (Fig. 10). Moreover, the CLASP2-p120-ECad complex was maintained under these conditions. How can we explain these results? Are MTs required for the proper formation of AJs and traffic of vesicles in broader terms?

The textbook idea of vesicle trafficking during exocytosis can be summarized in one sentence: MTs mediate the long-range transport of vesicles (from the Golgi towards their final destinations) whereas actin is implicated in the final stages, from arrival to the cortex to membrane delivery. In the reverse direction, during endocytosis, vesicles would first move on the cortical actin network associated to myosin, and dynein/kinesin MT motors would then mediate the long-range step (Ross et al., 2008). However, there is evidence for alternative mechanisms of transport (Schuh, 2011).

The involvement of MTs in AJs homeostasis has been broadly tested using MT depolymerizing drugs, such as colchicine or nocodazole. However, depolymerization of the MT network activates Rho (most likely via release of a sequestered GEF (Chang et al., 2008)) and inhibits Rac, which globally lead to an increase in actin contractility and stress fibers formation (Ory et al., 2002). This effect may impact on AJs formation and stability due to the fundamental role of actin for AJs homeostasis. In 1996, the group of J. Nelson reported that actin filaments were absolutely required for the formation of AJs in L cells expressing ECad (as observed after treatment with cytochalasin D); however, nocodazole treatment did not cause any alteration in AJs formation in this setting (Angres et al., 1996). On the contrary, treatment of PtK2 cells with nocodazole compromised AJs formation (Ligon and Holzbaur, 2007). Use of different nocodazole concentrations, incomplete MT depolymerization and/or cell type differences may explain the different effects observed.

Pointing towards a role for MTs in cadherin trafficking, NCad molecules have been observed to move towards cell-cell contact sites along MT tracks, associated to kinesin motors (Mary et al., 2002; Teng et al., 2005). Indeed, p120 has been proposed to mediate the link between NCad and kinesin as previously mentioned (Chen et al., 2003). Therefore, the importance of MTs for AJs formation may be cell-type specific and different cadherins may use different pathways for their traffic to the surface. As an example, VECad molecules have been shown to be transported by myosin motors (Almagro et al., 2010). In primary mKer, we favor the idea that MTs do not play a major role for the formation of AJs. Additionally, the effect of MT depolymerization could be related to a loss of polarized traffic of vesicles. Indeed MTs are required for the apical polarization of thyroid cells (Yap et al., 1995) and intestinal Caco-2 epithelial cells (Eilers et al., 1989) *in vitro.* In our culture system we did not distinguish different AJs domains, thus the role of MTs for polarized formation of AJs in keratinocytes remains to be further investigated.

Regarding the recruitment of CLASP2 to AJs in absence of MTs we observed an intial enrichment of CLASP2 at the membrane upon contact formation. However, whereas control cells progressively increased the levels of AJs-associated CLASP2, nocodazole treated cells did not show such an increase. This may indicate that a pool of CLASP2 is associated to cortical actin yet its arrival to cell-cell contacts via MTs is compromised.

2.2. Maintenance of Adherens Junctions

To address the role of MTs in the maintenance of AJs we treated mKer with well formed cell-cell contacts with nocodazole. Our results show that in absence of MTs, AJs are not disassembled (Fig. 8). On the contrary, after nocodazole treatment the levels of surface cadherins seemed to increase with time, suggesting that most likely cadherins were not internalized (Fig. 40). Supporting this hypothesis, when AJs where formed in cells lacking MTs, the initial steps of cell-cell contact formation were not affected, but at later time points of contact formation we observed a significant

increase in the levels of surface ECad when compared to control mKer (Fig. 10) This resembles the behavior observed at FAs, in which nocodazole treatment induces FA formation via RhoGTPase, and MT regrowth after nocodazole induces FA disassembly (Ezratty et al., 2005). Thus, it would be interesting to address the effect of MT regrowth after nocodazole on ECad levels at AJs.

Our results differ with two prior observations. Yap and col., using primary thyroid cell cultures, observed junctional discontinuities after depolymerization of the MT network with colchicine (Yap et al., 1995); and Waterman-Storer and col., obtained equivalent results upon treatment of primary newt lung epithelial cells with nocodazole (Waterman-Storer et al., 2000). These discrepancies may be due to differences in the cell context. Indeed, in MDCK cells treatment with nocodazole has no major effect on junctional ECad levels (Rosin-Arbesfeld et al., 2001), whereas MT depolymerization in human colonic epithelial cells (SK-CO-15) inhibits junction disassembly upon calcium removal, suggesting that MTs are required for cadherin endocytosis (Ivanov et al., 2006). It has also been shown that treatment of human keratinocytes with nocodazole in absence of calcium induces AJs formation through actin polymerization at junctional sites (Kee and Steinert, 2001). However, we have never observed junctional accumulation of ECad in our mKer after nocodazole treatment, in agreement with the fact that cadherin trans-interactions and stabilization at the membrane strongly depend on extracellular calcium (Shapiro and Weis, 2009).

In summary, treatment with nocodazole has been shown to either induce or inhibit (depending on the cellular system) AJs disassembly. Our results suggest that MTs are required for AJs internalization in primary mKer but not AJs formation.

Regarding CLASP2, depolymerization of MTs did not affect its localization to AJs, suggesting that it may be engaged in MT-independent interactions with AJs components, including p120, and possibly cortical actin and/or ACF7 (Fig. 40).

3. A novel role for CLASP2 at Adherens Junctions

Our results indicated a role for CLASP2 in the formation and maintenance of proper AJs dynamics. In absence of CLASP2, AJs do not properly form and p120 has altered dynamics at cell-cell contacts (Fig. 20, 21, 23). Although CLASP2 has been observed at cell-ECM adhesion sites, the role of CLASP2 at these sites in terms of stability of adhesion complexes has not been evaluated. Thus, to our knowledge this is the first time a role for CLASP2 in the maintenance of adhesion homeostasis is described. In line with our results, CLASP2 has been shown to be required for proper clustering of acetylcholine receptors at the neuromuscular junction via MT targeting to these cortical sites (Schmidt et al., 2012). In *Arabidopsis thaliana*, two recent reports demonstrate a role for CLASP in

cortical MT organization and polarized localization of auxin (plant growth hormone) carriers (Ambrose et al., 2013; Kakar et al., 2013). Interestingly, a recent screen in *Drosophila* identified NCad as novel potential CLASP interactor in line with our results (Long et al., 2013).

Our FRAP analysis show that in absence of CLASP2 the dynamics of p120 at cell-cell contacts were reduced (Fig. 23). This effect could be due to a decrease in p120 delivery to the membrane, or alternatively due to a decrease in p120 internalization/dynamics. With our current results we cannot rule out any of these two possibilities; since CLASP2-deficiency leads to a delay in AJs formation (Fig. 20, 21) and the nocodazole experiments reveal a possible role of MTs for cadherin internalization (Fig. 8). We should also take into account the known role of CLASP2 as a MT organizer at the Golgi (Efimov et al., 2007). The alterations in Golgi-derived MTs in absence of CLASP2 lead to a randomized vesicle trafficking without impacting the efficiency of transport (Miller et al., 2009). Thus, it would be interesting to evaluate whether absence of CLASP2 leads to alterations in the polarized trafficking of vesicle in the context of AJs formation.

Calcium removal led to a faster disassembly of cadherins in absence of CLASP2 (Fig. 28). Since absence of CLASP2 did not affect binding of p120 to ECad such a faster disassembly of cadherins is not a consequence of loss of p120 interaction with ECad (Fig. 22). On the contrary, it seems more likely that absence of CLASP2 impacts on the dynamics of AJs as a whole. Further prove for this hypothesis would require analysis of ECad dynamics by FRAP in CLASP2-deficient mKer.

Interestingly, it has been shown that treatment with low concentrations of nocodazole (which disrupts plus-end MT dynamics) does not affect ECad trafficking to the membrane but leads to a decrease mobility of ECad at cell adhesion sites. An equivalent phenotype is observed with a mutated form of CLIP-170. These results have been explained as the consequence of an inability to concentrate ECad at the membrane when MT dynamics are disrupted (Stehbens et al., 2006). Dynamic MTs are required to activate myosin II downstream of RhoA by delivering RhoGEFs to the AJs (Ratheesh et al., 2012). Due to the similarity of the phenotype observed upon CLASP2 knock-down and after inhibiting MT dynamics, we reasoned that the junctional defects were rooted to the alterations of the MT network observed in absence of either p120 or CLASP2. These experiments support a role for MTs, and specifically CLASP2, in the maintenance of proper AJs dynamics. Whether CLASP2 deficiency also affects the concentration and clustering of ECad at the membrane remains to be further explored.

However, there is a structural and functional crosstalk between MTs and actin filaments, making it virtually impossible to manipulate one without affecting the other (Rodriguez et al., 2003). As a matter of fact, CLASP2 is an actin binding protein (Tsvetkov et al., 2007), and thus, we cannot rule out the involvement of actin in the AJs phenotype observed. The functional relevance of such an interaction is currently unknown, and elucidating MT-specific or actin-specific CLASP2 roles is

complicated by the fact that the actin binding domain and the MT binding domain of CLASP2 almost completely overlap (Tsvetkov et al., 2007). In the future, it would be interesting to characterize with detail the actin network at AJs in absence of CLASP2.

3.1. Plus-ends as platforms for protein interactions at Adherens Junctions

CLASPs were described as CLIP interacting proteins that bind MT plus-ends in an EB1 dependent manner (Akhmanova et al., 2001; Mimori-Kiyosue et al., 2005). Additional studies have shown that *Drosophila* CLASP interacts with ACF7 (Long et al., 2013) and in mammalian cells CLASP2 functions downstream of ACF7 during polarized cell migration (Drabek et al., 2006) although colocalization between both proteins has not been tested in the same cell (Stehbens and Wittmann, 2012). CLASP2 also interacts with the Rac and Cdc42 effector IQGAP1 (Watanabe et al., 2009), which has fundamental roles during cell-cell contact formation (Noritake et al., 2005). APC and CLIP170 also interact with IQGAP1 (Galjart, 2010), but the functional relevance of all these interactions remains unknown (Stehbens and Wittmann, 2012). In addition, as mentioned previously, CLASPs, APC and ACF7 do not only bind MT plus-ends but also actin filaments. Which of these proteins has been studied in the context of AJs? And how does it fit in our model?

The CLIP-170-IQGAP1 interaction has been described as a mechanism for MT capture at the leading edge of migrating fibroblasts (Fukata et al., 2002). IQGAP1 also localizes to sites of cell-cell adhesion (Noritake et al., 2005), but whether the CLIP-170-IQGAP1 interaction mediates MT targeting to AJs has not been formally tested. IQGAP1 is known to bind directly β-catenin, inhibiting the β-catenin-α-catenin interaction, and leading to cell-cell dissociation. Thus, it is unlikely that the CLIP-170-IQGAP1 represents a mechanism of MT targeting to AJs. Another candidate is APC, which is found at AJs in both *Drosophila* and mammalian cells (Rosin-Arbesfeld et al., 2001; Townsley and Bienz, 2000). At these sites it seems to interact with cortical actin, but whether it mediates MT capture together with EB1 remains to be experimentally validated (Stehbens et al., 2009). ACF7 has also been observed at AJs in primary mKer but its function at this location remains to be investigated (Karakesisoglou et al., 2000). The most accepted mechanism for MT plus-end capture at AJs is the interaction between dynein and β-catenin described in PtK2 cells. It has been suggested that dynein tethers MTs to AJs in a β-catenin dependent manner (Ligon and Holzbaur, 2007; Ligon et al., 2001).

From the different potential mechanisms described, it would be interesting to further investigate whether ACF7 functions upstream of CLASP2 during MT targeting to AJs, in an equivalent manner to what has been observed in the leading edge of migrating cells (Drabek et al., 2006; Wu et al., 2008). Given the fact that MT depolymerization does not perturb CLASP2 localization to AJs, we hypothesize that CLASP2 could further interact with cortical proteins such as ACF7 or actin. Indeed our preliminary results showed colocalization between CLASP2 and cortical actin at AJs (data not

shown). The cortical localization of numerous +TIP proteins is not affected after depolymerization of the MT network with nocodazole. This is the case for ACF7 at AJs (Karakesisoglou et al., 2000), CLASPs at the leading edge of migrating cells (Drabek et al., 2006; Lansbergen et al., 2006; Mimori-Kiyosue et al., 2005), and APC both at cell-cell contacts and at the leading edge of migrating cells (Rosin-Arbesfeld et al., 2001; Zaoui et al., 2010). APC localization to AJs is more evident after depolymerization of the MT network, in an equivalent manner to what we have observed in our primary mKer (Fig. 8). It would be interesting to study whether APC and CLASP2 function together at the level of AJs, although in the leading edge of migrating PtK1 cells they were shown to localize to different subsets of MTs (Wittmann and Waterman-Storer, 2005). Thus, APC, ACF7 and CLASPs share their ability to bind both the actin and the MT cytoskeleton. Binding to the actin cytoskeleton may be especially relevant at cortical sites where these proteins may aid in MT targeting and tethering. From our own results and what has already been published, CLASPs, APC and ACF7 share a cortical MT-capture function both at cell-ECM and cell-cell adhesion sites.

We propose a model in which there are two pools of CLASP2 at AJs (Fig. 40): a MT plus-end-associated pool, and a MT-independent pool. The latter could be forming additional complexes with actin, ACF7, IQGAP1 or any other protein yet to be discovered, and this may be relevant for the proper organization and dynamics of junctional components. Such a model of actin-dependent and MT-dependent pools has already been described for APC and could represent a general feature of +TIP proteins that bind both actin and MT cytoskeletons (Rosin-Arbesfeld et al., 2001).

It is important to mention that we also observed CLASP1 localization at AJs in mKer (Fig. 13), but this localization did not depend on p120 presence. Therefore, other mechanisms may regulate CLASP1 targeting to sites of cell-cell adhesion. Whether CLASP1 also plays a role in the maintenance of AJs homeostasis remains to be addressed.

4. CLASP2 and p120: where do they find each other?

We have showed that CLASP2 and p120 interact at AJs (Fig. 7). But, do they interact at MT plus-ends before reaching cell-cell contacts? From our immunofluorescence experiments we did not detect colocalization at MT plus-ends and only a weak colocalization at the perinuclear region (which most likely corresponds to the Golgi) (Fig. 31c). However, the possibility still remains that CLASP2 associates with p120 at non-adhesion sites since CLASP2 and p120 coimmunoprecipitate in low calcium conditions in which AJs are not formed (Fig. 31a, 41). It remains to be addressed whether this complex also contains ECad.

The association between p120 and ECad during vesicular traffic is still controversial. Although most reports indicate that p120 may bind cadherins once they reach the surface (Xiao et al., 2007), a

recent publication showed that p120 binds to ECad at the Golgi (Curtis et al., 2008). Given the well-established role of CLASP2 at the Golgi (Efimov et al., 2007; Miller et al., 2009), an attractive hypothesis is the formation of a CLASP2-p120-ECad complex at the Golgi, which would be required for proper delivery of cadherins to the surface (Fig. 41). This could explain the delay in AJs formation we observed in absence of CLASP2 as well as the defect in AJs formation in p120-null mKer (Perez-Moreno et al., 2006). Interestingly, both p120-null and CLASP2-deficient mKer show reduced proliferation *in vitro* and spindle defects (Fig. 29) (Pereira et al., 2006; Perez-Moreno et al., 2008). These phenotypes could be partially a consequence of an altered interaction between both proteins, whether at centrosomes or at other subcellular locations such as mitotic spindles. In support of an interaction between CLASP2 and p120 at centrosomes, we could detect p120 together with CLASP2 in protein fractions containing purified centrosomes (Fig. 32). The functional relevance of this interaction remains to be further elucidated.

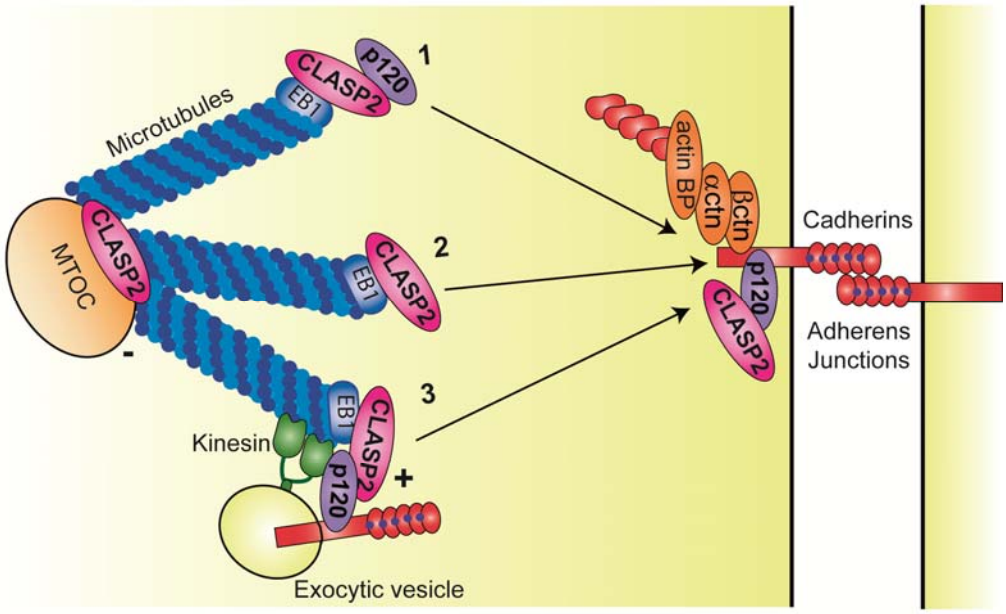


Figure 41 Possible models for the localization of the CLASP2-p120 interaction. CLASP2 and p120 may travel together at MT plus-ends to reach AJs sites (1). Alternatively, CLASP2 may interact with p120 upon reaching AJs, leading to MT targeting to cell-cell contacts (2). Since p120 is known to interact with kinesin motors, a p120-CLASP2 interaction at MTs may be relevant for the traffic of cadherins from the Golgi to the membrane. In this case a complex formed by CLASP2, p120, kinesin and vesicles containing cadherin molecules may form during exocytic trafficking (3).

5. CLASP2 and p120 interact in basal cells of the epidermis

One of the most relevant findings of this thesis project is the observation that CLASP2 is enriched in basal progenitor cells of the epidermis (Fig. 33). Differentiation of mKer *in vitro* leads to a decrease

in CLASP2 expression and absence of CLASP2 in mKer leads to premature differentiation (Fig. 30). Thus, CLASP2 may regulate epidermal homeostasis in different aspects beyond maintenance of AJs dynamics. Of note, ACF7 is enriched in hair follicle SCs where it plays a critical role for SC migration and wound healing (Wu et al., 2011). Since CLASP2 and ACF7 share a number of features, it would be interesting to study in more detail whether CLASP2 is also enriched in this SC compartment.

5.1. Epidermal organization

MTs in the basal layer of the epidermis present a centrosomal organization, with their plus-ends projecting towards areas of cell-cell contact (Lechler and Fuchs, 2007). We propose that this connection is mediated by the CLASP2-p120 interaction (Fig. 42). Upon differentiation, not only CLASP2 levels decrease, but also the MT network reorganizes, ninein relocalizes to desmosomes via interaction with desmoplakin and MTs are anchored at cell-cell adhesion sites (Lechler and Fuchs, 2007). Interestingly, we have seen that Nezha, a minus-end MT binding protein that interacts with p120 via PLEKHA7 (Meng et al., 2008), localizes to cell-cell contacts in suprabasal cells (Fig. 34). This observation led us to hypothesize that MT plus-ends preferentially associate to AJs in basal progenitor cells via the CLASP2-p120 interaction, whereas in suprabasal differentiated cells, minus-ends associate to cell-cell contacts together with Nezha and p120. Whether Nezha is recruited to desmosomes or remains associated to AJs in suprabasal epidermal cells remains to be addressed (Fig. 42). In broader terms, it remains to be analyzed whether MTs are targeted to AJs in suprabasal differentiated keratinocytes or they are exclusively anchored to desmosomes.

p120 deficient epidermis did not show any defects in CLASP2 or Nezha localization to sites of cell-cell adhesion (Fig. 35). It has previously been reported that absence of p120 in the epidermis does not lead to major alterations in AJs components, probably due to functional redundancy between p120 subfamily members (Perez-Moreno et al., 2006). Accordingly, we hypothesize that the lack of CLASP2 and Nezha alterations in p120 deficient epidermis may be due to partial compensation by ARVCF or other subfamily members. Alternatively, junctional cadherins could lead to the recruitment of CLASP2 and Nezha in a p120-independent manner, since *in vitro* we have seen that other mechanisms may cooperate to target CLASP2 to cell-cell contact sites.

Regarding CLIP-170, our results do not support the possibility that a CLASP2-CLIP-170 complex is formed at AJs in our system since CLIP-170 is expressed in suprabasal epidermal cells (Wacker et al., 1992). Interestingly, the +TIP protein LIS1, which binds CLIP-170 (Gouveia and Akhmanova, 2010), has been shown to localize to desmosomes in suprabasal mKer (Sumigray et al., 2011). Absence of LIS1 in the epidermis leads to altered MT organization in suprabasal mKer and desmosomal defects. Whether these phenotypes are also mediated by CLIP-170 remains to be explored. It should be noted that in the neuromuscular junction, CLASP2 has been shown to function together with CLIPs in targeting MTs to these sites (Schmidt et al., 2012). It would also be interesting to evaluate

CLASP1 localization in the epidermis and assess whether it presents a similar pattern of distribution as CLASP2.

5.2. Polarization and spindle orientation

The role of cadherins in spindle orientation and polarization of the centrosome has recently begun to be explored. Asymmetric cadherin ligation has been shown to play a role in centrosome positioning in rat kidney epithelial cells (Desai et al., 2009) and astrocytes (Dupin et al., 2009). These studies expand the role of cadherins from controlling apico-basal polarity in epithelial polarized cells to the establishment of polarity in otherwise non-polarized cells.

We have validated these findings in primary mKer grown in low confluency to allow the formation of small colonies. Asymmetric cell-cell contacts led to centrosome relocalization towards the junction. In addition, this relocalization did not take place in absence of p120 (data not shown). This novel role for cadherins in centrosome positioning may be extrapolated to mitotic cells. In *Drosophila* germ line stem cells (Inaba et al., 2010) and MDCK cells (den Elzen et al., 2009) ECad is required for proper spindle positioning, whereas in mouse epidermal progenitor cells, absence of α-catenin leads to spindle orientation defects (Lechler and Fuchs, 2005)

Given the fact that CLASP2 is enriched in basal epidermal cells, it is required for proper spindle formation and it localizes to areas of cell-cell contacts as well as centrosomes, we hypothesize that it may play a role in spindle orientation by interacting with cadherin-based adhesions. A recent report demonstrated the relevance of CLASP2 for the maintenance of hematopoietic SCs (Drabek et al., 2012). Mice deficient for CLASP2 present severe defects in hematopoiesis. The authors of the work hypothesize that CLASP2 is required for SCs to properly recognize their bone marrow niche. Our results expand this notion to the epidermis. However, we have not been able to validate such a role for CLASP2 in the maintenance of epidermal progenitor cells due to the technical limitation we have encounter during the generation of the CLASP2$^{\Delta ep}$ mouse model (Fig. 37, 38). In addition, a recent publication showed a role for CLASP1, and not CLASP2, in the polarized orientation of the spindle in HeLa cells (Samora et al., 2011). The functional relevance of a spindle orientation pathway in HeLa cells *in vitro* remains to be determined.

We hypothesize that CLASP2 may control the polarized orientation of the spindle during the divisions of epidermal progenitor cells, thus impacting on the balance between proliferation and differentiation (Fig. 42). This could explain the premature differentiation and decreased cell proliferation we have observed *in vitro*.

6. Relevance for human disease

The involvement of p120 in human tumorigenesis has been extensively documented (Thoreson and Reynolds, 2002). In certain circumstances, p120 can function as an oncogene by performing cadherin-independent functions such as inhibition of Kaiso in the nucleus or activation of Rac and Cdc42. In the first case, inhibition of Kaiso would lead to the activation of β-catenin target genes as well as genes implicated in metastasis and matrix remodeling. Indeed, Kaiso may be a tumor suppressor (Daniel, 2007). On the other hand, activation of Rac and Cdc42 would increase cell migration, although a direct link between these mechanisms and tumorigenesis has not been established (van Hengel and van Roy, 2007)

Well recognized is the function of p120 as a tumor suppressor. In this scenario loss of p120 would lead to ECad degradation (a hallmark of EMT) as well as to the development of an inflammatory disease via activation of NFκB (van Hengel and van Roy, 2007). The causal role for the loss of p120 in the development of carcinomas has been validated using conditional KO mouse models. Absence of p120 in the epidermis leads to the development of SCCs (Perez-Moreno et al., 2006), in the salivary gland loss of p120 induces neoplastic lesions (Davis and Reynolds, 2006), in the esophagus mice that lack p120 develop invasive SCCs (Stairs et al., 2011) and mice that lack p120 in the intestine die within 20 days due to inflammation, loss of cell-cell adhesion and intestinal bleeding (Smalley-Freed et al., 2012). It remains to be elucidated whether other known p120 functions such as: MT regulation, transcriptional regulation, or the novel p120-CLASP2 interaction have an impact on p120 tumor suppressor activity.

In this regard, we have observed a loss of CLASP2 expression in mouse skin carcinomas with different genetic backgrounds, including p120-deficient SCCs (Fig. 39). Different lines of human SCCs also display reductions in the total levels of CLASP2 (Fig. 39). Loss of CLASP2 may be a common feature of other types of carcinomas since in a high-throughput loss-of-heterozygosity study in human lung cancer CLASP2 was identified as a potential tumor suppressor gene (Tai et al., 2006). The concerted action of p120 and CLASP2 during tumorigenesis remains to be further explored. By means of regulating cell-cell adhesion and possibly spindle orientation and cell fate, the p120-CLASP2 interaction may be relevant for the maintenance of proper epidermal homeostasis.

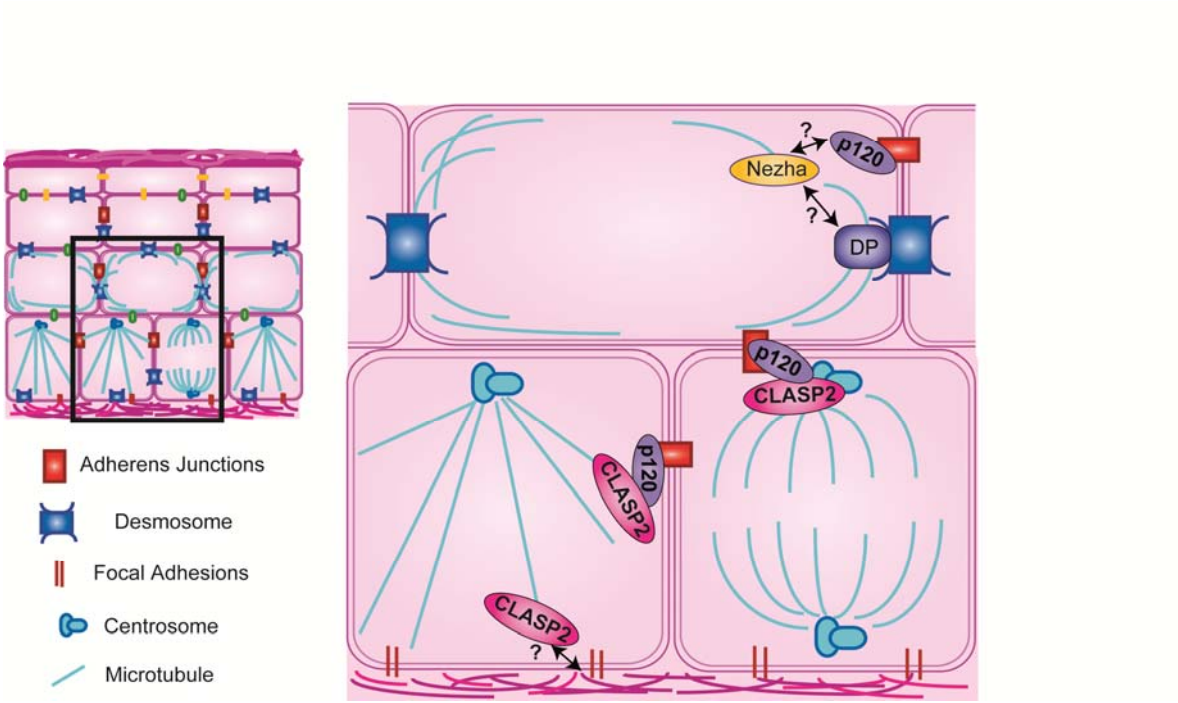


Figure 42 CLASP2-p120 interaction at AJs in basal progenitor cells of the epidermis. Our results support a model in which p120 and CLASP2 interact at AJs in basal cells of the epidermis, controlling AJs homeostasis and possibly spindle orientation. On the contrary, Nezha localizes to cell-cell contacts in suprabasal differentiated cells. Whether it interacts with AJs components or desmosomes remains to be explored.

Conclusions

1. CLASP2 and p120 interact at Adherens Junctions in a cadherin-dependent manner. Absence of p120 specifically impairs CLASP2 recruitment to cell-cell adhesion sites but does not affect CLASP2 localization at other sites. CLASP1 localizes to Adherens Junctions in a p120-independent manner.

2. CLASP2 and p120 directly interact via the N-terminal regulatory domain of p120 and the N1 region of CLASP2.

3. The interaction between CLASP2 and p120 at Adherens Junctions is maintained after disruption of microtubules, yet CLASP2-decorated microtubules are also observed at cadherin puncta. Thus, a microtubule-associated pool and a microtubule-independent pool of CLASP2 are present at Adherens Junctions.

4. CLASP2 can be recruited to cell-cell contacts in absence of microtubules. Alternative mechanisms of transport may exist to allow CLASP2 recruitment to cadherin-based adhesions.

5. CLASP2 deficiency in primary mouse keratinocytes does not affect the total levels of Adherens Junction proteins, yet it leads to a delay in the formation of cell-cell contacts. Moreover, the dynamic behavior of p120 at Adherens Junctions is impaired, leading to a global decrease in cell-cell adhesion.

6. Absence of either CLASP2 or p120 leads to a decrease in microtubule targeting to Adherens Junctions and alterations in the dynamic properties of microtubules at these sites. Microtubules do not properly pause when they reach an area of cadherins, present an increased growth rate and show a tendency towards aberrant trajectories.

7. CLASP2 deficiency in primary mouse keratinocytes leads to premature differentiation, decreased proliferation, decreased colony formation capabilities and spindle alterations. These features point towards possible alterations in positioning of centrosomes and mitotic spindles, and thus, cell fate.

8. CLASP2 expression is enriched in basal epidermal progenitor cells where it presents a cortical localization pattern suggestive of interactions with Adherens Junctions components. On the contrary, the minus-end microtubule binding protein Nezha localizes to cell-cell contacts in suprabasal cells. These results point towards a model in which MT plus-ends are targeted to Adherens Junctions in basal epidermal cells through the p120-CLASP2 interaction; whereas microtubule minus-ends localize to cell-cell contacts in suprabasal differentiated cells.

9. CLASP2 expression is decreased in mouse skin squamous cell carcinomas as well as in human squamous carcinoma cell lines.

Conclusiones

1. CLASP2 y p120 interaccionan en las Uniones Adherentes de forma dependiente de la presencia de cadherinas. La ausencia de p120 conduce a la pérdida de CLASP2 específicamente de las zonas de unión célula-célula, pero no afecta la localización de CLASP2 en otras estructuras celulares. La localización de CLASP1 en las uniones célula-célula no depende de la presencia de p120.

2. CLASP2 y p120 interaccionan de forma directa a través del dominio regulatorio N-terminal de p120 y la región N1 de CLASP2.

3. La interacción entre CLASP2 y p120 en las Uniones Adherentes se mantiene en ausencia de microtúbulos. Por otro lado, los extremos positivos de microtúbulos asociados a CLASP2 también se observan en zonas de unión mediada por las cadherinas. Por lo tanto, en las Uniones Adherentes coexisten moléculas de CLASP2 asociadas a microtúbulos y moléculas de CLASP2 no asociadas a microtúbulos.

4. CLASP2 puede reclutarse a las Uniones Adherentes en ausencia de microtúbulos, lo cual sugiere la existencia de mecanismos alternativos de transporte de CLASP2 a las zonas de unión entre células.

5. La ausencia de CLASP2 en queratinocitos primarios de ratón no afecta a los niveles totales de las proteínas de adhesión, pero retrasa la formación de las Uniones Adherentes. Además, la ausencia de CLASP2 altera el comportamiento dinámico de p120 en las Uniones Adherentes, lo cual conduce a una disminución global de la adhesión celular mediada por cadherinas.

6. La ausencia de tanto CLASP2 como de p120 conduce a una disminución de la asociación de los microtúbulos con las Uniones Adherentes y a alteraciones en la dinámica de los microtúbulos en las uniones entre células. Los microtúbulos no pausan correctamente al llegar a las Uniones Adherentes, presentan una mayor tasa de crecimiento y, en muchos casos, trayectorias aberrantes al llegar a las zonas de unión entre células.

7. La ausencia de CLASP2 en queratinocitos primarios de ratón conduce a una diferenciación celular prematura, una disminución de la capacidad de formación de colonias y alteraciones en el huso mitótico. Estas características fenotípicas indican posibles alteraciones en el posicionamiento de los centrosomas y los husos mitóticos, y por lo tanto, en el balance entre proliferación y diferenciación.

8. La expresión de CLASP2 está enriquecida en células basales progenitoras de la epidermis. En estas células CLASP2 presenta un patrón de localización cortical, lo cual sugiere la existencia de interacciones con los componentes de las Uniones Adherentes. Por el contrario, la proteína de

unión a los extremos negativos de los microtúbulos, Nezha, se localiza en las Uniones Adherentes de las células suprabasales. Estos resultados sugieren un modelo en el cual la asociación de los extremos positivos de los microtúbulos con las Uniones Adherentes en células basales de la epidermis se realiza a través de la interacción entre CLASP2 y p120; mientras que los extremos negativos de los microtúbulos se asocian con las Uniones Adherentes en células suprabasales de la epidermis.

9. La expresión de CLASP2 está disminuida en carcinomas escamosos de piel de ratón así como en líneas celulares de carcinomas escamosos de piel humanos.

References

Abedin, M., and N. King. 2008. The premetazoan ancestry of cadherins. *Science*. 319:946-8.
Abedin, M., and N. King. 2010. Diverse evolutionary paths to cell adhesion. *Trends Cell Biol*. 20:734-42.
Akhmanova, A., C.C. Hoogenraad, K. Drabek, T. Stepanova, B. Dortland, T. Verkerk, W. Vermeulen, B.M. Burgering, C.I. De Zeeuw, F. Grosveld, and N. Galjart. 2001. Clasps are CLIP-115 and -170 associating proteins involved in the regional regulation of microtubule dynamics in motile fibroblasts. *Cell*. 104:923-35.
Akhmanova, A., S.J. Stehbens, and A.S. Yap. 2009. Touch, grasp, deliver and control: functional cross-talk between microtubules and cell adhesions. *Traffic*. 10:268-74.
Akhmanova, A., and M.O. Steinmetz. 2008. Tracking the ends: a dynamic protein network controls the fate of microtubule tips. *Nat Rev Mol Cell Biol*. 9:309-22.
Akhmanova, A., and M.O. Steinmetz. 2010. Microtubule +TIPs at a glance. *J Cell Sci*. 123:3415-9.
Alema, S., and A.M. Salvatore. 2007. p120 catenin and phosphorylation: Mechanisms and traits of an unresolved issue. *Biochim Biophys Acta*. 1773:47-58.
Almagro, S., C. Durmort, A. Chervin-Petinot, S. Heyraud, M. Dubois, O. Lambert, C. Maillefaud, E. Hewat, J.P. Schaal, P. Huber, and D. Gulino-Debrac. 2010. The motor protein myosin-X transports VE-cadherin along filopodia to allow the formation of early endothelial cell-cell contacts. *Mol Cell Biol*. 30:1703-17.
Ambrose, C., Y. Ruan, J. Gardiner, L.M. Tamblyn, A. Catching, V. Kirik, J. Marc, R. Overall, and G.O. Wasteneys. 2013. CLASP Interacts with Sorting Nexin 1 to Link Microtubules and Auxin Transport via PIN2 Recycling in Arabidopsis thaliana. *Dev Cell*. 24:649-59.
Ambrose, J.C., and G.O. Wasteneys. 2008. CLASP modulates microtubule-cortex interaction during self-organization of acentrosomal microtubules. *Mol Biol Cell*. 19:4730-7.
Amos, L.A., and D. Schlieper. 2005. Microtubules and maps. *Adv Protein Chem*. 71:257-98.
Anastasiadis, P.Z. 2007. p120-ctn: A nexus for contextual signaling via Rho GTPases. *Biochim Biophys Acta*. 1773:34-46.
Anastasiadis, P.Z., S.Y. Moon, M.A. Thoreson, D.J. Mariner, H.C. Crawford, Y. Zheng, and A.B. Reynolds. 2000. Inhibition of RhoA by p120 catenin. *Nat Cell Biol*. 2:637-44.
Anastasiadis, P.Z., and A.B. Reynolds. 2000. The p120 catenin family: complex roles in adhesion, signaling and cancer. *J Cell Sci*. 113 (Pt 8):1319-34.
Angres, B., A. Barth, and W.J. Nelson. 1996. Mechanism for transition from initial to stable cell-cell adhesion: kinetic analysis of E-cadherin-mediated adhesion using a quantitative adhesion assay. *J Cell Biol*. 134:549-57.
Baki, L., P. Marambaud, S. Efthimiopoulos, A. Georgakopoulos, P. Wen, W. Cui, J. Shioi, E. Koo, M. Ozawa, V.L. Friedrich, Jr., and N.K. Robakis. 2001. Presenilin-1 binds cytoplasmic epithelial cadherin, inhibits cadherin/p120 association, and regulates stability and function of the cadherin/catenin adhesion complex. *Proc Natl Acad Sci U S A*. 98:2381-6.
Bartolini, F., and G.G. Gundersen. 2006. Generation of noncentrosomal microtubule arrays. *J Cell Sci*. 119:4155-63.
Baum, B., and M. Georgiou. 2011. Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. *J Cell Biol*. 192:907-17.
Bellett, G., J.M. Carter, J. Keynton, D. Goldspink, C. James, D.K. Moss, and M.M. Mogensen. 2009. Microtubule plus-end and minus-end capture at adherens junctions is involved in the assembly of apico-basal arrays in polarised epithelial cells. *Cell Motil Cytoskeleton*.
Bienz, M. 2005. beta-Catenin: a pivot between cell adhesion and Wnt signalling. *Curr Biol*. 15:R64-7.
Blanpain, C., and E. Fuchs. 2009. Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat Rev Mol Cell Biol*. 10:207-17.
Borisy, G.G., and E.W. Taylor. 1967. The mechanism of action of colchicine. Binding of colchincine-3H to cellular protein. *J Cell Biol*. 34:525-33.
Brinkley, W. 1997. Microtubules: a brief historical perspective. *J Struct Biol*. 118:84-6.

Bryant, D.M., and J.L. Stow. 2004. The ins and outs of E-cadherin trafficking. *Trends Cell Biol*. 14:427-34.

Bugnard, E., K.J. Zaal, and E. Ralston. 2005. Reorganization of microtubule nucleation during muscle differentiation. *Cell Motil Cytoskeleton*. 60:1-13.

Bulinski, J.C., and G.G. Gundersen. 1991. Stabilization of post-translational modification of microtubules during cellular morphogenesis. *Bioessays*. 13:285-93.

Burbank, K.S., and T.J. Mitchison. 2006. Microtubule dynamic instability. *Curr Biol*. 16:R516-7.

Campbell, I.D., and M.J. Humphries. 2011. Integrin structure, activation, and interactions. *Cold Spring Harb Perspect Biol*. 3.

Candi, E., R. Schmidt, and G. Melino. 2005. The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol*. 6:328-40.

Cano, A., M.A. Perez-Moreno, I. Rodrigo, A. Locascio, M.J. Blanco, M.G. del Barrio, F. Portillo, and M.A. Nieto. 2000. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol*. 2:76-83.

Caviston, J.P., and E.L. Holzbaur. 2006. Microtubule motors at the intersection of trafficking and transport. *Trends Cell Biol*. 16:530-7.

Chang, Y.C., P. Nalbant, J. Birkenfeld, Z.F. Chang, and G.M. Bokoch. 2008. GEF-H1 couples nocodazole-induced microtubule disassembly to cell contractility via RhoA. *Mol Biol Cell*. 19:2147-53.

Chappuis-Flament, S., E. Wong, L.D. Hicks, C.M. Kay, and B.M. Gumbiner. 2001. Multiple cadherin extracellular repeats mediate homophilic binding and adhesion. *J Cell Biol*. 154:231-43.

Chartier, N.T., C.I. Oddou, M.G. Laine, B. Ducarouge, C.A. Marie, M.R. Block, and M.R. Jacquier-Sarlin. 2007. Cyclin-dependent kinase 2/cyclin E complex is involved in p120 catenin (p120ctn)-dependent cell growth control: a new role for p120ctn in cancer. *Cancer Res*. 67:9781-90.

Chausovsky, A., A.D. Bershadsky, and G.G. Borisy. 2000. Cadherin-mediated regulation of microtubule dynamics. *Nat Cell Biol*. 2:797-804.

Cheeseman, I.M., I. MacLeod, J.R. Yates, 3rd, K. Oegema, and A. Desai. 2005. The CENP-F-like proteins HCP-1 and HCP-2 target CLASP to kinetochores to mediate chromosome segregation. *Curr Biol*. 15:771-7.

Chen, X., S. Kojima, G.G. Borisy, and K.J. Green. 2003. p120 catenin associates with kinesin and facilitates the transport of cadherin-catenin complexes to intercellular junctions. *J Cell Biol*. 163:547-57.

Curtis, M.W., K.R. Johnson, and M.J. Wheelock. 2008. E-cadherin/catenin complexes are formed cotranslationally in the endoplasmic reticulum/Golgi compartments. *Cell Commun Adhes*. 15:365-78.

Dammermann, A., A. Desai, and K. Oegema. 2003. The minus end in sight. *Curr Biol*. 13:R614-24.

Daniel, J.M. 2007. Dancing in and out of the nucleus: p120(ctn) and the transcription factor Kaiso. *Biochim Biophys Acta*. 1773:59-68.

Daniel, J.M., and A.B. Reynolds. 1999. The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. *Mol Cell Biol*. 19:3614-23.

Davis, M.A., R.C. Ireton, and A.B. Reynolds. 2003. A core function for p120-catenin in cadherin turnover. *J Cell Biol*. 163:525-34.

Davis, M.A., and A.B. Reynolds. 2006. Blocked acinar development, E-cadherin reduction, and intraepithelial neoplasia upon ablation of p120-catenin in the mouse salivary gland. *Dev Cell*. 10:21-31.

Dejana, E., E. Tournier-Lasserve, and B.M. Weinstein. 2009. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Dev Cell*. 16:209-21.

den Elzen, N., C.V. Buttery, M.P. Maddugoda, G. Ren, and A.S. Yap. 2009. Cadherin adhesion receptors orient the mitotic spindle during symmetric cell division in mammalian epithelia. *Mol Biol Cell*. 20:3740-50.

Desai, R.A., L. Gao, S. Raghavan, W.F. Liu, and C.S. Chen. 2009. Cell polarity triggered by cell-cell adhesion via E-cadherin. *J Cell Sci*. 122:905-11.

Dickinson, D.J., W.J. Nelson, and W.I. Weis. A polarized epithelium organized by beta- and alpha-catenin predates cadherin and metazoan origins. *Science*. 331:1336-9.

Drabek, K., L. Gutierrez, M. Vermeij, T. Clapes, S.R. Patel, J.C. Boisset, J. van Haren, A.L. Pereira, Z. Liu, U. Akinci, T. Nikolic, W. van Ijcken, M. van den Hout, M. Meinders, C. Melo, C. Sambade, D. Drabek, R.W. Hendriks, S. Philipsen, M. Mommaas, F. Grosveld, H. Maiato, J.E. Italiano, Jr., C. Robin, and N. Galjart. 2012. The microtubule plus-end tracking protein CLASP2 is required for hematopoiesis and hematopoietic stem cell maintenance. *Cell Rep*. 2:781-8.

Drabek, K., M. van Ham, T. Stepanova, K. Draegestein, R. van Horssen, C.L. Sayas, A. Akhmanova, T. Ten Hagen, R. Smits, R. Fodde, F. Grosveld, and N. Galjart. 2006. Role of CLASP2 in microtubule stabilization and the regulation of persistent motility. *Curr Biol*. 16:2259-64.

Drees, F., S. Pokutta, S. Yamada, W.J. Nelson, and W.I. Weis. 2005. Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell*. 123:903-15.

Dupin, I., E. Camand, and S. Etienne-Manneville. 2009. Classical cadherins control nucleus and centrosome position and cell polarity. *J Cell Biol*. 185:779-86.

Efimov, A., A. Kharitonov, N. Efimova, J. Loncarek, P.M. Miller, N. Andreyeva, P. Gleeson, N. Galjart, A.R. Maia, I.X. McLeod, J.R. Yates, 3rd, H. Maiato, A. Khodjakov, A. Akhmanova, and I. Kaverina. 2007. Asymmetric CLASP-dependent nucleation of noncentrosomal microtubules at the trans-Golgi network. *Dev Cell*. 12:917-30.

Eilers, U., J. Klumperman, and H.P. Hauri. 1989. Nocodazole, a microtubule-active drug, interferes with apical protein delivery in cultured intestinal epithelial cells (Caco-2). *J Cell Biol*. 108:13-22.

Erck, C., L. Peris, A. Andrieux, C. Meissirel, A.D. Gruber, M. Vernet, A. Schweitzer, Y. Saoudi, H. Pointu, C. Bosc, P.A. Salin, D. Job, and J. Wehland. 2005. A vital role of tubulin-tyrosine-ligase for neuronal organization. *Proc Natl Acad Sci U S A*. 102:7853-8.

Etienne-Manneville, S., and A. Hall. 2002. Rho GTPases in cell biology. *Nature*. 420:629-35.

Ezratty, E.J., M.A. Partridge, and G.G. Gundersen. 2005. Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat Cell Biol*. 7:581-90.

Farquhar, M.G., and G.E. Palade. 1963. Junctional complexes in various epithelia. *J Cell Biol*. 17:375-412.

Filler, R.B., S.J. Roberts, and M. Girardi. 2007. Cutaneous two-stage chemical carcinogenesis. *CSH Protoc*. 2007:pdb prot4837.

Franke, W.W. 2009. Discovering the molecular components of intercellular junctions--a historical view. *Cold Spring Harb Perspect Biol*. 1:a003061.

Franz, C.M., and A.J. Ridley. 2004. p120 catenin associates with microtubules: inverse relationship between microtubule binding and Rho GTPase regulation. *J Biol Chem*. 279:6588-94.

Fuchs, E. 2007. Scratching the surface of skin development. *Nature*. 445:834-42.

Fuchs, E., and V. Horsley. 2008. More than one way to skin. *Genes Dev*. 22:976-85.

Fuchs, E., and S. Raghavan. 2002. Getting under the skin of epidermal morphogenesis. *Nat Rev Genet*. 3:199-209.

Fujita, Y., G. Krause, M. Scheffner, D. Zechner, H.E. Leddy, J. Behrens, T. Sommer, and W. Birchmeier. 2002. Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat Cell Biol*. 4:222-31.

Fukata, M., T. Watanabe, J. Noritake, M. Nakagawa, M. Yamaga, S. Kuroda, Y. Matsuura, A. Iwamatsu, F. Perez, and K. Kaibuchi. 2002. Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170. *Cell*. 109:873-85.

Galjart, N. 2005. CLIPs and CLASPs and cellular dynamics. *Nat Rev Mol Cell Biol*. 6:487-98.
Galjart, N. 2010. Plus-end-tracking proteins and their interactions at microtubule ends. *Curr Biol*. 20:R528-37.
Gennerich, A., and R.D. Vale. 2009. Walking the walk: how kinesin and dynein coordinate their steps. *Curr Opin Cell Biol*. 21:59-67.
Gouveia, S.M., and A. Akhmanova. 2010. Cell and molecular biology of microtubule plus end tracking proteins: end binding proteins and their partners. *Int Rev Cell Mol Biol*. 285:1-74.
Green, K.J., and J.C. Jones. 1996. Desmosomes and hemidesmosomes: structure and function of molecular components. *FASEB J*. 10:871-81.
Green, K.J., and C.L. Simpson. 2007. Desmosomes: new perspectives on a classic. *J Invest Dermatol*. 127:2499-515.
Grimson, M.J., J.C. Coates, J.P. Reynolds, M. Shipman, R.L. Blanton, and A.J. Harwood. 2000. Adherens junctions and beta-catenin-mediated cell signalling in a non-metazoan organism. *Nature*. 408:727-31.
Gumbiner, B., B. Stevenson, and A. Grimaldi. 1988. The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J Cell Biol*. 107:1575-87.
Gumbiner, B.M. 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell*. 84:345-57.
Gumbiner, B.M. 2005. Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol*. 6:622-34.
Gundersen, G.G. 2002. Evolutionary conservation of microtubule-capture mechanisms. *Nat Rev Mol Cell Biol*. 3:296-304.
Gundersen, G.G., E.R. Gomes, and Y. Wen. 2004. Cortical control of microtubule stability and polarization. *Curr Opin Cell Biol*. 16:106-12.
Gundersen, G.G., S. Khawaja, and J.C. Bulinski. 1987. Postpolymerization detyrosination of alpha-tubulin: a mechanism for subcellular differentiation of microtubules. *J Cell Biol*. 105:251-64.
Hagios, C., A. Lochter, and M.J. Bissell. 1998. Tissue architecture: the ultimate regulator of epithelial function? *Philos Trans R Soc Lond B Biol Sci*. 353:857-70.
Halbleib, J.M., and W.J. Nelson. 2006. Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev*. 20:3199-214.
Hanahan, D., and R.A. Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell*. 144:646-74.
Hannak, E., and R. Heald. 2006. Xorbit/CLASP links dynamic microtubules to chromosomes in the Xenopus meiotic spindle. *J Cell Biol*. 172:19-25.
Harris, T.J., and U. Tepass. 2010. Adherens junctions: from molecules to morphogenesis. *Nat Rev Mol Cell Biol*. 11:502-14.
Hermiston, M.L., M.H. Wong, and J.I. Gordon. 1996. Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for nonautonomous regulation of cell fate in a self-renewing system. *Genes Dev*. 10:985-96.
Hinck, L., I.S. Nathke, J. Papkoff, and W.J. Nelson. 1994. Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. *J Cell Biol*. 125:1327-40.
Hotta, A., T. Kawakatsu, T. Nakatani, T. Sato, C. Matsui, T. Sukezane, T. Akagi, T. Hamaji, I. Grigoriev, A. Akhmanova, Y. Takai, and Y. Mimori-Kiyosue. 2010. Laminin-based cell adhesion anchors microtubule plus ends to the epithelial cell basal cortex through LL5alpha/beta. *J Cell Biol*. 189:901-17.
Huelsken, J., R. Vogel, B. Erdmann, G. Cotsarelis, and W. Birchmeier. 2001. beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell*. 105:533-45.
Hyafil, F., C. Babinet, and F. Jacob. 1981. Cell-cell interactions in early embryogenesis: a molecular approach to the role of calcium. *Cell*. 26:447-54.

Ichii, T., and M. Takeichi. 2007. p120-catenin regulates microtubule dynamics and cell migration in a cadherin-independent manner. *Genes Cells*. 12:827-39.

Inaba, M., H. Yuan, V. Salzmann, M.T. Fuller, and Y.M. Yamashita. 2010. E-cadherin is required for centrosome and spindle orientation in Drosophila male germline stem cells. *PLoS One*. 5:e12473.

Ireton, R.C., M.A. Davis, J. van Hengel, D.J. Mariner, K. Barnes, M.A. Thoreson, P.Z. Anastasiadis, L. Matrisian, L.M. Bundy, L. Sealy, B. Gilbert, F. van Roy, and A.B. Reynolds. 2002. A novel role for p120 catenin in E-cadherin function. *J Cell Biol*. 159:465-76.

Ishiyama, N., S.H. Lee, S. Liu, G.Y. Li, M.J. Smith, L.F. Reichardt, and M. Ikura. 2010. Dynamic and static interactions between p120 catenin and E-cadherin regulate the stability of cell-cell adhesion. *Cell*. 141:117-28.

Ivanov, A.I., I.C. McCall, B. Babbin, S.N. Samarin, A. Nusrat, and C.A. Parkos. 2006. Microtubules regulate disassembly of epithelial apical junctions. *BMC Cell Biol*. 7:12.

Jamora, C., R. DasGupta, P. Kocieniewski, and E. Fuchs. 2003. Links between signal transduction, transcription and adhesion in epithelial bud development. *Nature*. 422:317-22.

Jiang, K., and A. Akhmanova. 2011. Microtubule tip-interacting proteins: a view from both ends. *Curr Opin Cell Biol*. 23:94-101.

Kakar, K., H. Zhang, B. Scheres, and P. Dhonukshe. 2013. CLASP-mediated cortical microtubule organization guides PIN polarization axis. *Nature*. 495:529-33.

Kamath, K., E. Oroudjev, and M.A. Jordan. Determination of microtubule dynamic instability in living cells. *Methods Cell Biol*. 97:1-14.

Karakesisoglou, I., Y. Yang, and E. Fuchs. 2000. An epidermal plakin that integrates actin and microtubule networks at cellular junctions. *J Cell Biol*. 149:195-208.

Kartenbeck, J., M. Schmelz, W.W. Franke, and B. Geiger. 1991. Endocytosis of junctional cadherins in bovine kidney epithelial (MDBK) cells cultured in low Ca2+ ion medium. *J Cell Biol*. 113:881-92.

Keating, T.J., and G.G. Borisy. 1999. Centrosomal and non-centrosomal microtubules. *Biol Cell*. 91:321-9.

Kee, S.H., and P.M. Steinert. 2001. Microtubule disruption in keratinocytes induces cell-cell adhesion through activation of endogenous E-cadherin. *Mol Biol Cell*. 12:1983-93.

Kirschner, M., and T. Mitchison. 1986. Beyond self-assembly: from microtubules to morphogenesis. *Cell*. 45:329-42.

Kitagawa, D., I. Vakonakis, N. Olieric, M. Hilbert, D. Keller, V. Olieric, M. Bortfeld, M.C. Erat, I. Fluckiger, P. Gonczy, and M.O. Steinmetz. 2011. Structural basis of the 9-fold symmetry of centrioles. *Cell*. 144:364-75.

Kobielak, A., and E. Fuchs. 2006. Links between alpha-catenin, NF-kappaB, and squamous cell carcinoma in skin. *Proc Natl Acad Sci U S A*. 103:2322-7.

Kodama, A., I. Karakesisoglou, E. Wong, A. Vaezi, and E. Fuchs. 2003. ACF7: an essential integrator of microtubule dynamics. *Cell*. 115:343-54.

Komarova, Y.A., F. Huang, M. Geyer, N. Daneshjou, A. Garcia, L. Idalino, B. Kreutz, D. Mehta, and A.B. Malik. 2012. VE-cadherin signaling induces EB3 phosphorylation to suppress microtubule growth and assemble adherens junctions. *Mol Cell*. 48:914-25.

Kumar, P., K.S. Lyle, S. Gierke, A. Matov, G. Danuser, and T. Wittmann. 2009. GSK3beta phosphorylation modulates CLASP-microtubule association and lamella microtubule attachment. *J Cell Biol*. 184:895-908.

Kumar, P., and T. Wittmann. 2012. +TIPs: SxIPping along microtubule ends. *Trends Cell Biol*. 22:418-28.

Lansbergen, G., I. Grigoriev, Y. Mimori-Kiyosue, T. Ohtsuka, S. Higa, I. Kitajima, J. Demmers, N. Galjart, A.B. Houtsmuller, F. Grosveld, and A. Akhmanova. 2006. CLASPs attach microtubule plus ends to the cell cortex through a complex with LL5beta. *Dev Cell*. 11:21-32.

Lanza, D.C., G.V. Meirelles, M.R. Alborghetti, C.H. Abrile, G. Lenz, and J. Kobarg. FEZ1 interacts with CLASP2 and NEK1 through coiled-coil regions and their cellular colocalization suggests centrosomal functions and regulation by PKC. *Mol Cell Biochem*. 338:35-45.

Larue, L., M. Ohsugi, J. Hirchenhain, and R. Kemler. 1994. E-cadherin null mutant embryos fail to form a trophectoderm epithelium. *Proc Natl Acad Sci U S A*. 91:8263-7.

Lechler, T., and E. Fuchs. 2005. Asymmetric cell divisions promote stratification and differentiation of mammalian skin. *Nature*. 437:275-80.

Lechler, T., and E. Fuchs. 2007. Desmoplakin: an unexpected regulator of microtubule organization in the epidermis. *J Cell Biol*. 176:147-54.

Ligon, L.A., and E.L. Holzbaur. 2007. Microtubules tethered at epithelial cell junctions by dynein facilitate efficient junction assembly. *Traffic*. 8:808-19.

Ligon, L.A., S. Karki, M. Tokito, and E.L. Holzbaur. 2001. Dynein binds to beta-catenin and may tether microtubules at adherens junctions. *Nat Cell Biol*. 3:913-7.

Lo, S.H. 2006. Focal adhesions: what's new inside. *Dev Biol*. 294:280-91.

Logarinho, E., S. Maffini, M. Barisic, A. Marques, A. Toso, P. Meraldi, and H. Maiato. 2012. CLASPs prevent irreversible multipolarity by ensuring spindle-pole resistance to traction forces during chromosome alignment. *Nat Cell Biol*. 14:295-303.

Long, J.B., M. Bagonis, L.A. Lowery, H. Lee, G. Danuser, and D. Van Vactor. 2013. Multiparametric Analysis of CLASP-Interacting Protein Functions during Interphase Microtubule Dynamics. *Mol Cell Biol*. 33:1528-45.

Louie, R.K., S. Bahmanyar, K.A. Siemers, V. Votin, P. Chang, T. Stearns, W.J. Nelson, and A.I. Barth. 2004. Adenomatous polyposis coli and EB1 localize in close proximity of the mother centriole and EB1 is a functional component of centrosomes. *J Cell Sci*. 117:1117-28.

Lowery, L.A., H. Lee, C. Lu, R. Murphy, R.A. Obar, B. Zhai, M. Schedl, D. Van Vactor, and Y. Zhan. 2010. Parallel genetic and proteomic screens identify Msps as a CLASP-Abl pathway interactor in Drosophila. *Genetics*. 185:1311-25.

Luders, J., and T. Stearns. 2007. Microtubule-organizing centres: a re-evaluation. *Nat Rev Mol Cell Biol*. 8:161-7.

Maia, A.R., Z. Garcia, L. Kabeche, M. Barisic, S. Maffini, S. Macedo-Ribeiro, I.M. Cheeseman, D.A. Compton, I. Kaverina, and H. Maiato. 2012. Cdk1 and Plk1 mediate a CLASP2 phospho-switch that stabilizes kinetochore-microtubule attachments. *J Cell Biol*. 199:285-301.

Maiato, H., E.A. Fairley, C.L. Rieder, J.R. Swedlow, C.E. Sunkel, and W.C. Earnshaw. 2003. Human CLASP1 is an outer kinetochore component that regulates spindle microtubule dynamics. *Cell*. 113:891-904.

Maiato, H., A. Khodjakov, and C.L. Rieder. 2005. Drosophila CLASP is required for the incorporation of microtubule subunits into fluxing kinetochore fibres. *Nat Cell Biol*. 7:42-7.

Maiato, H., P. Sampaio, C.L. Lemos, J. Findlay, M. Carmena, W.C. Earnshaw, and C.E. Sunkel. 2002. MAST/Orbit has a role in microtubule-kinetochore attachment and is essential for chromosome alignment and maintenance of spindle bipolarity. *J Cell Biol*. 157:749-60.

Malikov, V., A. Kashina, and V. Rodionov. 2004. Cytoplasmic dynein nucleates microtubules to organize them into radial arrays in vivo. *Mol Biol Cell*. 15:2742-9.

Marambaud, P., J. Shioi, G. Serban, A. Georgakopoulos, S. Sarner, V. Nagy, L. Baki, P. Wen, S. Efthimiopoulos, Z. Shao, T. Wisniewski, and N.K. Robakis. 2002. A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *EMBO J*. 21:1948-56.

Martin-Belmonte, F., and M. Perez-Moreno. 2012. Epithelial cell polarity, stem cells and cancer. *Nat Rev Cancer*. 12:23-38.

Mary, S., S. Charrasse, M. Meriane, F. Comunale, P. Travo, A. Blangy, and C. Gauthier-Rouviere. 2002. Biogenesis of N-cadherin-dependent cell-cell contacts in living fibroblasts is a microtubule-dependent kinesin-driven mechanism. *Mol Biol Cell*. 13:285-301.

Matsumoto, S., K. Fumoto, T. Okamoto, K. Kaibuchi, and A. Kikuchi. 2010. Binding of APC and dishevelled mediates Wnt5a-regulated focal adhesion dynamics in migrating cells. *EMBO J*. 29:1192-204.

McCrea, P.D., and J.I. Park. 2007. Developmental functions of the P120-catenin sub-family. *Biochim Biophys Acta*. 1773:17-33.

Meigs, T.E., and D.D. Kaplan. 2008. Isolation of centrosomes from cultured Mammalian cells. *CSH Protoc*. 2008:pdb prot5039.

Meng, W., Y. Mushika, T. Ichii, and M. Takeichi. 2008. Anchorage of microtubule minus ends to adherens junctions regulates epithelial cell-cell contacts. *Cell*. 135:948-59.

Meng, W., and M. Takeichi. 2009. Adherens junction: molecular architecture and regulation. *Cold Spring Harb Perspect Biol*. 1:a002899.

Mese, G., G. Richard, and T.W. White. 2007. Gap junctions: basic structure and function. *J Invest Dermatol*. 127:2516-24.

Michels, C., T. Buchta, W. Bloch, T. Krieg, and C.M. Niessen. 2009. Classical cadherins regulate desmosome formation. *J Invest Dermatol*. 129:2072-5.

Miller, P.M., A.W. Folkmann, A.R. Maia, N. Efimova, A. Efimov, and I. Kaverina. 2009. Golgi-derived CLASP-dependent microtubules control Golgi organization and polarized trafficking in motile cells. *Nat Cell Biol*. 11:1069-80.

Mimori-Kiyosue, Y., I. Grigoriev, G. Lansbergen, H. Sasaki, C. Matsui, F. Severin, N. Galjart, F. Grosveld, I. Vorobjev, S. Tsukita, and A. Akhmanova. 2005. CLASP1 and CLASP2 bind to EB1 and regulate microtubule plus-end dynamics at the cell cortex. *J Cell Biol*. 168:141-53.

Mimori-Kiyosue, Y., I. Grigoriev, H. Sasaki, C. Matsui, A. Akhmanova, S. Tsukita, and I. Vorobjev. 2006. Mammalian CLASPs are required for mitotic spindle organization and kinetochore alignment. *Genes Cells*. 11:845-57.

Mimori-Kiyosue, Y., and S. Tsukita. 2001. Where is APC going? *J Cell Biol*. 154:1105-9.

Miranda, K.C., S.R. Joseph, A.S. Yap, R.D. Teasdale, and J.L. Stow. 2003. Contextual binding of p120ctn to E-cadherin at the basolateral plasma membrane in polarized epithelia. *J Biol Chem*. 278:43480-8.

Miyashita, Y., and M. Ozawa. 2007. Increased internalization of p120-uncoupled E-cadherin and a requirement for a dileucine motif in the cytoplasmic domain for endocytosis of the protein. *J Biol Chem*. 282:11540-8.

Morrison, E.E. 2007. Action and interactions at microtubule ends. *Cell Mol Life Sci*. 64:307-17.

Musch, A. 2004. Microtubule organization and function in epithelial cells. *Traffic*. 5:1-9.

Musil, L.S., B.A. Cunningham, G.M. Edelman, and D.A. Goodenough. 1990. Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J Cell Biol*. 111:2077-88.

Nagafuchi, A., Y. Shirayoshi, K. Okazaki, K. Yasuda, and M. Takeichi. 1987. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature*. 329:341-3.

Nanes, B.A., C. Chiasson-MacKenzie, A.M. Lowery, N. Ishiyama, V. Faundez, M. Ikura, P.A. Vincent, and A.P. Kowalczyk. 2012. p120-catenin binding masks an endocytic signal conserved in classical cadherins. *J Cell Biol*. 199:365-80.

Navarro, P., M. Gomez, A. Pizarro, C. Gamallo, M. Quintanilla, and A. Cano. 1991. A role for the E-cadherin cell-cell adhesion molecule during tumor progression of mouse epidermal carcinogenesis. *J Cell Biol*. 115:517-33.

Niessen, C.M. 2007. Tight junctions/adherens junctions: basic structure and function. *J Invest Dermatol*. 127:2525-32.

Niessen, C.M., D. Leckband, and A.S. Yap. 2011. Tissue organization by cadherin adhesion molecules: dynamic molecular and cellular mechanisms of morphogenetic regulation. *Physiol Rev*. 91:691-731.

Nogales, E. 2000. Structural insights into microtubule function. *Annu Rev Biochem*. 69:277-302.

Noritake, J., T. Watanabe, K. Sato, S. Wang, and K. Kaibuchi. 2005. IQGAP1: a key regulator of adhesion and migration. *J Cell Sci*. 118:2085-92.

Nowak, J.A., and E. Fuchs. 2009. Isolation and culture of epithelial stem cells. *Methods Mol Biol*. 482:215-32.

Ocana, O.H., R. Corcoles, A. Fabra, G. Moreno-Bueno, H. Acloque, S. Vega, A. Barrallo-Gimeno, A. Cano, and M.A. Nieto. 2012. Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prrx1. *Cancer Cell*. 22:709-24.

Ory, S., O. Destaing, and P. Jurdic. 2002. Microtubule dynamics differentially regulates Rho and Rac activity and triggers Rho-independent stress fiber formation in macrophage polykaryons. *Eur J Cell Biol*. 81:351-62.

Ozawa, M., H. Baribault, and R. Kemler. 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J*. 8:1711-7.

Palazzo, A.F., T.A. Cook, A.S. Alberts, and G.G. Gundersen. 2001. mDia mediates Rho-regulated formation and orientation of stable microtubules. *Nat Cell Biol*. 3:723-9.

Peifer, M., and A.S. Yap. 2003. Traffic control: p120-catenin acts as a gatekeeper to control the fate of classical cadherins in mammalian cells. *J Cell Biol*. 163:437-40.

Peinado, H., F. Portillo, and A. Cano. 2004. Transcriptional regulation of cadherins during development and carcinogenesis. *Int J Dev Biol*. 48:365-75.

Pereira, A.L., A.J. Pereira, A.R. Maia, K. Drabek, C.L. Sayas, P.J. Hergert, M. Lince-Faria, I. Matos, C. Duque, T. Stepanova, C.L. Rieder, W.C. Earnshaw, N. Galjart, and H. Maiato. 2006. Mammalian CLASP1 and CLASP2 cooperate to ensure mitotic fidelity by regulating spindle and kinetochore function. *Mol Biol Cell*. 17:4526-42.

Perez-Moreno, M., M.A. Davis, E. Wong, H.A. Pasolli, A.B. Reynolds, and E. Fuchs. 2006. p120-catenin mediates inflammatory responses in the skin. *Cell*. 124:631-44.

Perez-Moreno, M., and E. Fuchs. 2006. Catenins: keeping cells from getting their signals crossed. *Dev Cell*. 11:601-12.

Perez-Moreno, M., C. Jamora, and E. Fuchs. 2003. Sticky business: orchestrating cellular signals at adherens junctions. *Cell*. 112:535-48.

Perez-Moreno, M., W. Song, H.A. Pasolli, S.E. Williams, and E. Fuchs. 2008. Loss of p120 catenin and links to mitotic alterations, inflammation, and skin cancer. *Proc Natl Acad Sci U S A*. 105:15399-404.

Perez-Moreno, M.A., A. Locascio, I. Rodrigo, G. Dhondt, F. Portillo, M.A. Nieto, and A. Cano. 2001. A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions. *J Biol Chem*. 276:27424-31.

Perez, F., G.S. Diamantopoulos, R. Stalder, and T.E. Kreis. 1999. CLIP-170 highlights growing microtubule ends in vivo. *Cell*. 96:517-27.

Peris, L., M. Thery, J. Faure, Y. Saoudi, L. Lafanechere, J.K. Chilton, P. Gordon-Weeks, N. Galjart, M. Bornens, L. Wordeman, J. Wehland, A. Andrieux, and D. Job. 2006. Tubulin tyrosination is a major factor affecting the recruitment of CAP-Gly proteins at microtubule plus ends. *J Cell Biol*. 174:839-49.

Perl, A.K., P. Wilgenbus, U. Dahl, H. Semb, and G. Christofori. 1998. A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature*. 392:190-3.

Poulson, N.D., and T. Lechler. 2010. Robust control of mitotic spindle orientation in the developing epidermis. *J Cell Biol*. 191:915-22.

Quyn, A.J., P.L. Appleton, F.A. Carey, R.J. Steele, N. Barker, H. Clevers, R.A. Ridgway, O.J. Sansom, and I.S. Nathke. 2010. Spindle orientation bias in gut epithelial stem cell compartments is lost in precancerous tissue. *Cell Stem Cell*. 6:175-81.

Ramirez, A., A. Bravo, J.L. Jorcano, and M. Vidal. 1994. Sequences 5' of the bovine keratin 5 gene direct tissue- and cell-type-specific expression of a lacZ gene in the adult and during development. *Differentiation*. 58:53-64.

Ratheesh, A., G.A. Gomez, R. Priya, S. Verma, E.M. Kovacs, K. Jiang, N.H. Brown, A. Akhmanova, S.J. Stehbens, and A.S. Yap. 2012. Centralspindlin and alpha-catenin regulate Rho signalling at the epithelial zonula adherens. *Nat Cell Biol*. 14:818-28.

Ray, S., and T. Lechler. 2011. Regulation of asymmetric cell division in the epidermis. *Cell Div*. 6:12.

Reilein, A., and W.J. Nelson. 2005. APC is a component of an organizing template for cortical microtubule networks. *Nat Cell Biol*. 7:463-73.

Reynolds, A.B., and R.H. Carnahan. 2004. Regulation of cadherin stability and turnover by p120ctn: implications in disease and cancer. *Semin Cell Dev Biol*. 15:657-63.

Reynolds, A.B., J. Daniel, P.D. McCrea, M.J. Wheelock, J. Wu, and Z. Zhang. 1994. Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes. *Mol Cell Biol*. 14:8333-42.

Reynolds, A.B., D.J. Roesel, S.B. Kanner, and J.T. Parsons. 1989. Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cells expressing oncogenic variants of the avian cellular src gene. *Mol Cell Biol*. 9:629-38.

Richard, G. 2000. Connexins: a connection with the skin. *Exp Dermatol*. 9:77-96.

Roczniak-Ferguson, A., and A.B. Reynolds. 2003. Regulation of p120-catenin nucleocytoplasmic shuttling activity. *J Cell Sci*. 116:4201-12.

Rodriguez, O.C., A.W. Schaefer, C.A. Mandato, P. Forscher, W.M. Bement, and C.M. Waterman-Storer. 2003. Conserved microtubule-actin interactions in cell movement and morphogenesis. *Nat Cell Biol*. 5:599-609.

Rosin-Arbesfeld, R., G. Ihrke, and M. Bienz. 2001. Actin-dependent membrane association of the APC tumour suppressor in polarized mammalian epithelial cells. *EMBO J*. 20:5929-39.

Ross, J.L., M.Y. Ali, and D.M. Warshaw. 2008. Cargo transport: molecular motors navigate a complex cytoskeleton. *Curr Opin Cell Biol*. 20:41-7.

Samora, C.P., B. Mogessie, L. Conway, J.L. Ross, A. Straube, and A.D. McAinsh. 2011. MAP4 and CLASP1 operate as a safety mechanism to maintain a stable spindle position in mitosis. *Nat Cell Biol*. 13:1040-50.

Schatten, H. 2008. The mammalian centrosome and its functional significance. *Histochem Cell Biol*. 129:667-86.

Schmidt, N., S. Basu, S. Sladecek, S. Gatti, J. van Haren, S. Treves, J. Pielage, N. Galjart, and H.R. Brenner. 2012. Agrin regulates CLASP2-mediated capture of microtubules at the neuromuscular junction synaptic membrane. *J Cell Biol*. 198:421-37.

Schuh, M. 2011. An actin-dependent mechanism for long-range vesicle transport. *Nat Cell Biol*. 13:1431-6.

Schulze, E., and M. Kirschner. 1986. Microtubule dynamics in interphase cells. *J Cell Biol*. 102:1020-31.

Schuyler, S.C., and D. Pellman. 2001. Microtubule "plus-end-tracking proteins": The end is just the beginning. *Cell*. 105:421-4.

Schwartz, M. 2004. Rho signalling at a glance. *J Cell Sci*. 117:5457-8.

Shapiro, L., and W.I. Weis. 2009. Structure and biochemistry of cadherins and catenins. *Cold Spring Harb Perspect Biol*. 1:a003053.

Shaw, R.M., A.J. Fay, M.A. Puthenveedu, M. von Zastrow, Y.N. Jan, and L.Y. Jan. 2007. Microtubule plus-end-tracking proteins target gap junctions directly from the cell interior to adherens junctions. *Cell*. 128:547-60.

Shtutman, M., A. Chausovsky, M. Prager-Khoutorsky, N. Schiefermeier, S. Boguslavsky, Z. Kam, E. Fuchs, B. Geiger, G.G. Borisy, and A.D. Bershadsky. 2008. Signaling function of alpha-catenin in microtubule regulation. *Cell Cycle*. 7:2377-83.

Simpson, C.L., D.M. Patel, and K.J. Green. 2011. Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. *Nat Rev Mol Cell Biol*. 12:565-80.

Slep, K.C. 2009. The role of TOG domains in microtubule plus end dynamics. *Biochem Soc Trans*. 37:1002-6.

Slep, K.C., and R.D. Vale. 2007. Structural basis of microtubule plus end tracking by XMAP215, CLIP-170, and EB1. *Mol Cell*. 27:976-91.

Smalley-Freed, W.G., A. Efimov, P.E. Burnett, S.P. Short, M.A. Davis, D.L. Gumucio, M.K. Washington, R.J. Coffey, and A.B. Reynolds. 2012. p120-catenin is essential for maintenance of barrier function and intestinal homeostasis in mice. *J Clin Invest*. 120:1824-35.

Sonnenberg, A., J. Calafat, H. Janssen, H. Daams, L.M. van der Raaij-Helmer, R. Falcioni, S.J. Kennel, J.D. Aplin, J. Baker, M. Loizidou, and et al. 1991. Integrin alpha 6/beta 4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion. *J Cell Biol*. 113:907-17.

Stairs, D.B., L.J. Bayne, B. Rhoades, M.E. Vega, T.J. Waldron, J. Kalabis, A. Klein-Szanto, J.S. Lee, J.P. Katz, J.A. Diehl, A.B. Reynolds, R.H. Vonderheide, and A.K. Rustgi. 2011. Deletion of p120-catenin results in a tumor microenvironment with inflammation and cancer that establishes it as a tumor suppressor gene. *Cancer Cell*. 19:470-83.

Stehbens, S., and T. Wittmann. 2012. Targeting and transport: how microtubules control focal adhesion dynamics. *J Cell Biol*. 198:481-9.

Stehbens, S.J., A. Akhmanova, and A.S. Yap. 2009. Microtubules and cadherins: a neglected partnership. *Front Biosci*. 14:3159-67.

Stehbens, S.J., A.D. Paterson, M.S. Crampton, A.M. Shewan, C. Ferguson, A. Akhmanova, R.G. Parton, and A.S. Yap. 2006. Dynamic microtubules regulate the local concentration of E-cadherin at cell-cell contacts. *J Cell Sci*. 119:1801-11.

Stepanova, T., J. Slemmer, C.C. Hoogenraad, G. Lansbergen, B. Dortland, C.I. De Zeeuw, F. Grosveld, G. van Cappellen, A. Akhmanova, and N. Galjart. 2003. Visualization of microtubule growth in cultured neurons via the use of EB3-GFP (end-binding protein 3-green fluorescent protein). *J Neurosci*. 23:2655-64.

Stiess, M., N. Maghelli, L.C. Kapitein, S. Gomis-Ruth, M. Wilsch-Brauninger, C.C. Hoogenraad, I.M. Tolic-Norrelykke, and F. Bradke. 2010. Axon extension occurs independently of centrosomal microtubule nucleation. *Science*. 327:704-7.

Sumigray, K.D., H. Chen, and T. Lechler. 2011. Lis1 is essential for cortical microtubule organization and desmosome stability in the epidermis. *J Cell Biol*. 194:631-42.

Sumigray, K.D., H.P. Foote, and T. Lechler. 2012. Noncentrosomal microtubules and type II myosins potentiate epidermal cell adhesion and barrier formation. *J Cell Biol*. 199:513-25.

Summers, K., and M.W. Kirschner. 1979. Characteristics of the polar assembly and disassembly of microtubules observed in vitro by darkfield light microscopy. *J Cell Biol*. 83:205-17.

Sun, T.T., and H. Green. 1976. Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope. *Cell*. 9:511-21.

Tachibana, K., H. Nakanishi, K. Mandai, K. Ozaki, W. Ikeda, Y. Yamamoto, A. Nagafuchi, S. Tsukita, and Y. Takai. 2000. Two cell adhesion molecules, nectin and cadherin, interact through their cytoplasmic domain-associated proteins. *J Cell Biol*. 150:1161-76.

Tai, A.L., W. Mak, P.K. Ng, D.T. Chua, M.Y. Ng, L. Fu, K.K. Chu, Y. Fang, Y. Qiang Song, M. Chen, M. Zhang, P.C. Sham, and X.Y. Guan. 2006. High-throughput loss-of-heterozygosity study of chromosome 3p in lung cancer using single-nucleotide polymorphism markers. *Cancer Res*. 66:4133-8.

Takai, Y., W. Ikeda, H. Ogita, and Y. Rikitake. 2008. The immunoglobulin-like cell adhesion molecule nectin and its associated protein afadin. *Annu Rev Cell Dev Biol*. 24:309-42.

Takeichi, M. 1977. Functional correlation between cell adhesive properties and some cell surface proteins. *J Cell Biol*. 75:464-74.

Teng, J., T. Rai, Y. Tanaka, Y. Takei, T. Nakata, M. Hirasawa, A.B. Kulkarni, and N. Hirokawa. 2005. The KIF3 motor transports N-cadherin and organizes the developing neuroepithelium. *Nat Cell Biol*. 7:474-82.

Thoreson, M.A., and A.B. Reynolds. 2002. Altered expression of the catenin p120 in human cancer: implications for tumor progression. *Differentiation*. 70:583-9.

Tinkle, C.L., T. Lechler, H.A. Pasolli, and E. Fuchs. 2004. Conditional targeting of E-cadherin in skin: insights into hyperproliferative and degenerative responses. *Proc Natl Acad Sci U S A*. 101:552-7.

Tinkle, C.L., H.A. Pasolli, N. Stokes, and E. Fuchs. 2008. New insights into cadherin function in epidermal sheet formation and maintenance of tissue integrity. *Proc Natl Acad Sci U S A*. 105:15405-10.

Togashi, H., K. Abe, A. Mizoguchi, K. Takaoka, O. Chisaka, and M. Takeichi. 2002. Cadherin regulates dendritic spine morphogenesis. *Neuron*. 35:77-89.

Torres, M., A. Stoykova, O. Huber, K. Chowdhury, P. Bonaldo, A. Mansouri, S. Butz, R. Kemler, and P. Gruss. 1997. An alpha-E-catenin gene trap mutation defines its function in preimplantation development. *Proc Natl Acad Sci U S A*. 94:901-6.

Townsley, F.M., and M. Bienz. 2000. Actin-dependent membrane association of a Drosophila epithelial APC protein and its effect on junctional Armadillo. *Curr Biol*. 10:1339-48.

Tsvetkov, A.S., A. Samsonov, A. Akhmanova, N. Galjart, and S.V. Popov. 2007. Microtubule-binding proteins CLASP1 and CLASP2 interact with actin filaments. *Cell Motil Cytoskeleton*. 64:519-30.

Tunggal, J.A., I. Helfrich, A. Schmitz, H. Schwarz, D. Gunzel, M. Fromm, R. Kemler, T. Krieg, and C.M. Niessen. 2005. E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. *EMBO J*. 24:1146-56.

Tyler, S. 2003. Epithelium--the primary building block for metazoan complexity. *Integr Comp Biol*. 43:55-63.

van Hengel, J., and F. van Roy. 2007. Diverse functions of p120ctn in tumors. *Biochim Biophys Acta*. 1773:78-88.

Vasioukhin, V., C. Bauer, L. Degenstein, B. Wise, and E. Fuchs. 2001. Hyperproliferation and defects in epithelial polarity upon conditional ablation of alpha-catenin in skin. *Cell*. 104:605-17.

Vasioukhin, V., C. Bauer, M. Yin, and E. Fuchs. 2000. Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell*. 100:209-19.

Vestweber, D., and R. Kemler. 1984. Rabbit antiserum against a purified surface glycoprotein decompacts mouse preimplantation embryos and reacts with specific adult tissues. *Exp Cell Res*. 152:169-78.

Wacker, I.U., J.E. Rickard, J.R. De Mey, and T.E. Kreis. 1992. Accumulation of a microtubule-binding protein, pp170, at desmosomal plaques. *J Cell Biol*. 117:813-24.

Wahl, J.K., 3rd, Y.J. Kim, J.M. Cullen, K.R. Johnson, and M.J. Wheelock. 2003. N-cadherin-catenin complexes form prior to cleavage of the proregion and transport to the plasma membrane. *J Biol Chem*. 278:17269-76.

Watabe, M., A. Nagafuchi, S. Tsukita, and M. Takeichi. 1994. Induction of polarized cell-cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma line. *J Cell Biol*. 127:247-56.

Watanabe, T., J. Noritake, M. Kakeno, T. Matsui, T. Harada, S. Wang, N. Itoh, K. Sato, K. Matsuzawa, A. Iwamatsu, N. Galjart, and K. Kaibuchi. 2009. Phosphorylation of CLASP2 by GSK-3beta regulates its interaction with IQGAP1, EB1 and microtubules. *J Cell Sci*. 122:2969-79.

Waterman-Storer, C.M., W.C. Salmon, and E.D. Salmon. 2000. Feedback interactions between cell-cell adherens junctions and cytoskeletal dynamics in newt lung epithelial cells. *Mol Biol Cell*. 11:2471-83.

Westermann, S., and K. Weber. 2003. Post-translational modifications regulate microtubule function. *Nat Rev Mol Cell Biol*. 4:938-47.

Williams, S.E., S. Beronja, H.A. Pasolli, and E. Fuchs. 2011. Asymmetric cell divisions promote Notch-dependent epidermal differentiation. *Nature*. 470:353-8.

Wittmann, T., and C.M. Waterman-Storer. 2005. Spatial regulation of CLASP affinity for microtubules by Rac1 and GSK3beta in migrating epithelial cells. *J Cell Biol*. 169:929-39.

Wu, X., A. Kodama, and E. Fuchs. 2008. ACF7 regulates cytoskeletal-focal adhesion dynamics and migration and has ATPase activity. *Cell*. 135:137-48.

Wu, X., Q.T. Shen, D.S. Oristian, C.P. Lu, Q. Zheng, H.W. Wang, and E. Fuchs. 2011. Skin stem cells orchestrate directional migration by regulating microtubule-ACF7 connections through GSK3beta. *Cell*. 144:341-52.

Xiao, K., R.G. Oas, C.M. Chiasson, and A.P. Kowalczyk. 2007. Role of p120-catenin in cadherin trafficking. *Biochim Biophys Acta*. 1773:8-16.

Yanagisawa, M., I.N. Kaverina, A. Wang, Y. Fujita, A.B. Reynolds, and P.Z. Anastasiadis. 2004. A novel interaction between kinesin and p120 modulates p120 localization and function. *J Biol Chem*. 279:9512-21.

Yap, A.S., B.R. Stevenson, K.C. Abel, E.J. Cragoe, Jr., and S.W. Manley. 1995. Microtubule integrity is necessary for the epithelial barrier function of cultured thyroid cell monolayers. *Exp Cell Res*. 218:540-50.

Zaoui, K., K. Benseddik, P. Daou, D. Salaun, and A. Badache. 2010. ErbB2 receptor controls microtubule capture by recruiting ACF7 to the plasma membrane of migrating cells. *Proc Natl Acad Sci U S A*. 107:18517-22.